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11. Document ID: AU 9952744 A WO 200009152 A1

L30: Entry 11 of 67

File: DWPI

Mar 6, 2000

DERWENT-ACC-NO: 2000-224175

DERWENT-WEEK: 200030

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TITLE: Therapeutic composition containing CXCR4 antagonist, useful for treating autoimmune disease, especially multiple sclerosis and cancer

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments	Claims	KWIC
Drawn Desc	Image										

- ☐
12. Document ID: MX 2001001426 A1 WO 200008139 A1 AU 9925934 A EP 1100886 A1 CN 1322244 A KR 2001085351 A

L30: Entry 12 of 67

File: DWPI

Aug 1, 2001

DERWENT-ACC-NO: 2000-205704

DERWENT-WEEK: 200238

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TITLE: New human tumor necrosis factor gamma-alpha and beta polypeptides useful for treating tumor or cancer via gene therapy, and for treating rheumatoid arthritis, liver cirrhosis, osteoarthritis and diabetes

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments	Claims	KWIC
Drawn Desc	Image										

- ☐
13. Document ID: CN 1317047 A WO 200005375 A1 AU 9951257 A EP 1098978 A1

L30: Entry 13 of 67

File: DWPI

Oct 10, 2001

DERWENT-ACC-NO: 2000-205377

DERWENT-WEEK: 200207

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TITLE: New polynucleotide encoding mammalian receptor for streptococcus toxin, useful for diagnosis and treatment of, e.g. pneumonia in neonates

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments	Claims	KWIC
Drawn Desc	Image										

- ☐
14. Document ID: AU 9944401 A WO 200005356 A1

L30: Entry 14 of 67

File: DWPI

Feb 14, 2000

DERWENT-ACC-NO: 2000-182688
DERWENT-WEEK: 200029
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TITLE: New anti-angiogenic protein containing an IGF binding, Willebrand factor type C, thrombospondin type 1 and cysteine knot domains is useful for inhibiting atopic angiogenesis e.g. in solid tumors

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments	Claims	KWIC
Draw Desc	Image										

☐ 15. Document ID: JP 2002520367 W WO 200003726 A1 AU 9949718 A EP 1096945 A1 MX 2000012085 A1

L30: Entry 15 of 67

File: DWPI

Jul 9, 2002

DERWENT-ACC-NO: 2000-171200
DERWENT-WEEK: 200259
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TITLE: Novel lysine binding fragments angiostatin used as antiangiogenic agents in the treatment of cancer, diabetic retinopathy, rheumatoid arthritis, psoriasis, atherosclerotic plaque formation, and other angiogenesis diseases

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments	Claims	KWIC
Draw Desc	Clip Img	Image									

☐ 16. Document ID: WO 200002902 A1 AU 9950979 A EP 1097165 A1

L30: Entry 16 of 67

File: DWPI

Jan 20, 2000

DERWENT-ACC-NO: 2000-171128
DERWENT-WEEK: 200015
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TITLE: Saposin B derived peptides, useful as inhibitors of angiogenesis and tumor growth

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments	KWIC
Draw Desc	Clip Img	Image								

☐ 17. Document ID: EP 962530 A2 WO 9962925 A1 AU 9943204 A

L30: Entry 17 of 67

File: DWPI

Dec 8, 1999

DERWENT-ACC-NO: 2000-025636
DERWENT-WEEK: 200003
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TITLE: New substantially pure Scarface 1 (Sf-1) protein, useful for screening compounds for modulating angiogenesis, to enhance wound healing, inhibit tumor growth and/or to amplify stem cells

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments
Draw Desc	Image								

KIMC

☐ 18. Document ID: EP 1082126 A1 WO 9961041 A1 AU 9942031 A

L30: Entry 18 of 67

File: DWPI

Mar 14, 2001

DERWENT-ACC-NO: 2000-062582

DERWENT-WEEK: 200116

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TITLE: New peptides corresponding to fibrinogen carboxy terminus, used for promoting wound healing

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments
Draw Desc	Image								

KIMC

☐ 19. Document ID: EP 1086956 A1 WO 9960025 A1 AU 9938503 A

L30: Entry 19 of 67

File: DWPI

Mar 28, 2001

DERWENT-ACC-NO: 2000-072431

DERWENT-WEEK: 200118

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TITLE: Gene recombinant antibodies, useful for diagnosis and as remedies for diseases due to abnormal neovascularization e.g. proliferation or metastasis of solid tumor, rheumatoid arthritis, diabetic retinopathy and psoriasis

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments
Draw Desc	Clip Img	Image							

KIMC

☐ 20. Document ID: EP 1073679 A1 WO 9954356 A1 AU 9936168 A

L30: Entry 20 of 67

File: DWPI

Feb 7, 2001

DERWENT-ACC-NO: 2000-013229

DERWENT-WEEK: 200109

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TITLE: New peptide derived from murine epidermal growth factor (mEGF)

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments
Draw Desc	Image								

KIMC

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L30: Entry 16 of 67

File: DWPI

Jan 20, 2000

DERWENT-ACC-NO: 2000-171128

DERWENT-WEEK: 200015

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TITLE: Saposin B derived peptides, useful as inhibitors of angiogenesis and tumor growth

Publication Date (1):20000120Publication Date (2):20000201Basic Abstract Text (1):

NOVELTY - Saposin B was found to have potent anti-angiogenic and antitumoral activity, as well as antiproliferative and anti-migratory activity against endothelial cells. These activities are conserved in cryptic polypeptides as small as 5 amino acids.

Basic Abstract Text (2):

DETAILED DESCRIPTION - An isolated polypeptide (I) of 5-71 amino acids and comprising a contiguous amino acid sequence:

Basic Abstract Text (6):

(1) a protein which specifically binds to Saposin B and is found on the surface of KS Y-1, SLK and HUVEC cells;

Basic Abstract Text (8):

(3) an isolated fusion protein comprising (I) and a cell targeting moiety, where (I) and the cell targeting moiety have functional activity independent of each other; and

Basic Abstract Text (12):

USE - The polypeptides are of use in the treatment of undesired angiogenesis and tumor growth, especially Kaposi's sarcoma (claimed). They can also be used in conjunction with cell cytotoxic moieties to selectively kill certain cell types. Diseases and pathological conditions treated include cancer, angiofibroma, neovascular glaucoma, arteriovenous malformations, nonunion fractures, arthritis and other connective tissue disorders, Osler-Weber syndrome, atherosclerotic plaques, psoriasis, corneal graft neovascularization, pyogenic granuloma, retrolental fibroplasia, diabetic retinopathy, scleroderma, hemangioma, trachoma, vascular adhesions and hypertrophic scars. The peptides have use in veterinary medicine to treat cats, dogs, horses and cattle, as well as humans.

Basic Abstract Text (14):

DESCRIPTION OF DRAWING(S) - Activity of recombinant Saposin B. Only Kaposi's syndrome and endothelial cells showed dose dependent growth inhibition.

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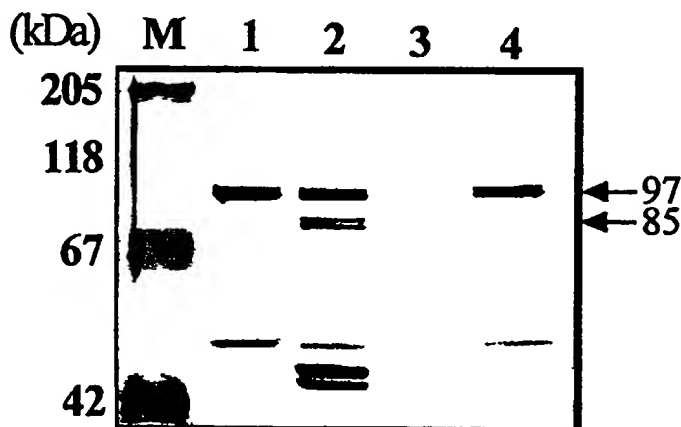
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[Suite sur la page suivante]

(54) Title: ICBP90 POLYPEPTIDE AND ITS FRAGMENTS AND POLYNUCLEOTIDES CODING FOR SAID POLYPEPTIDES
AND APPLICATIONS FOR DIAGNOSING AND TREATING CANCER

(54) Titre: POLYPEPTIDE ICBP90 ET SES FRAGMENTS ET POLYNUCLEOTIDES CODANT LESDITS POLYPEPTIDES ET
APPLICATIONS AU DIAGNOSTIC ET AU TRAITEMENT DU CANCER



(57) Abstract: The invention concerns a novel ICBP90 (Inverted CCAAT box binding protein 90) and its fragments, polynucleotides coding for said polypeptides and specific antibodies directed against said polypeptides. The invention also concerns methods and kits for diagnosing cell proliferation and compounds useful as medicine for preventing and/or treating pathology involving cell proliferation and in particular cancer.

(57) Abrégé: L'invention concerne un nouveau polypeptide ICBP90 (Inverted CCAAT box binding protein 90) et ses fragments, les polynucléotides codant pour lesdits polypeptides et des anticorps spécifiques dirigés contre lesdits

polypeptides. L'invention concerne également des procédés et des kits de diagnostic de prolifération cellulaire et des composés utilisables à titre de médicament pour la prévention et/ou le traitement de pathologie faisant intervenir la prolifération cellulaire et du cancer en particulier.



WO 00/78949 A1



En ce qui concerne les codes à deux lettres et autres abréviations, se référer aux "Notes explicatives relatives aux codes et abréviations" figurant au début de chaque numéro ordinaire de la Gazette du PCT.

« POLYPEPTIDE ICBP90 ET SES FRAGMENTS ET
POLYNUCLEOTIDES CODANT LESDITS POLYPEPTIDES ET
APPLICATIONS AU DIAGNOSTIC ET AU TRAITEMENT DU
CANCER ».

5 La présente invention concerne un nouveau polypeptide ICBP90 et ses fragments, le clonage de l'ADNc et les polynucléotides codant pour lesdits polypeptides, des vecteurs de clonage et/ou d'expression incluant lesdits polynucléotides, des cellules transformées par lesdits vecteurs et des anticorps spécifiques
10 dirigés contre lesdits polypeptides. L'invention concerne également des procédés et des kits de diagnostic des cancers, un procédé et un kit de criblage de ligands du polypeptide de l'invention et des composés utilisables à titre de médicament pour la prévention et/ou le traitement des cancers.

15 Les ADN topoisomérases sont des protéines nucléaires hautement conservées au cours l'évolution dont le rôle principal est de contrôler la conformation et la topologie de l'ADN dans le noyau, qui sont constamment altérées par les différents processus biologiques impliquant l'ADN tels par exemple la transcription et la
20 réplication. Les topoisomérases exercent leur action en coupant l'ADN et en reliant ces lésions après avoir réalisé le changement conformationnel adéquat.

 Chez les mammifères et l'homme en particulier, il existe à l'heure actuelle au moins cinq gènes différents codant pour une
25 topoisomérase et au moins deux pseudogènes additionnels (pour revue, voir Nitiss 1998). Ainsi, la topoisomérase I, codée par le gène TOP1 retire les supertours présents dans l'ADN en ne coupant qu'un seul brin. Les deux topoisomérases de type II existant chez l'homme appelées TopII α et TopII β , altèrent la topologie de l'ADN en
30 introduisant des clivages double brin transitoires (pour revue, voir

Wang 1996). Enfin, il existe deux topoisomérases de type III codées par deux gènes localisés en 17p11.2-12 et 22q11-12 et qui agissent uniquement contre les supertours négatifs de l'ADN.

Dans les cellules tumorales, les topoisomérases de type II jouent un rôle très important ; dans ces cellules en croissance et en division rapide, il existe un grand besoin de maintenir les molécules d'ADN dans une conformation correcte puisque des taux de transcription et de réplication élevés sont nécessaires. Ainsi, les taux de topoisomérases II sont en général plus élevés dans les cellules tumorales humaines que dans les tissus normaux de même origine. Cependant, le taux d'expression élevé de la topoisomérase II α dans les cellules tumorales peut varier entre deux tumeurs de nature différente affectant un même tissu. Par exemple, le noyau des cellules de carcinome du poumon à petites cellules présente un taux plus élevé de topoisomérase II α que le noyau des cellules de carcinomes pulmonaires à cellules de taille normale (Guinee *et al.*, 1996). De la même manière, le taux de topoisomérase II α dans les cellules A549 est trois fois plus élevé que dans les cellules PC3, ces deux lignées cellulaires provenant d'adénocarcinome de l'épithélium pulmonaire (Yamasaki *et al.*, 1996).

Ces constatations donnent à penser que la topoisomérase II α peut être considérée comme un marqueur de prolifération cellulaire pour certains types de cancer. Le processus cancéreux se caractérisant par une prolifération cellulaire anormale due en partie à la perte de l'inhibition de contact, la topoisomérase II α apparaît donc comme une cible privilégiée des drogues chimiothérapeutiques pour le traitement du cancer (Pommier *et al.* 1994), et les traitements anticancéreux actuels font largement appel aux inhibiteurs de topoisomérases.

La plupart de ces inhibiteurs exercent leurs effets cytotoxiques en stabilisant le complexe de clivage de l'ADN. Des drogues comme les anthracyclines [doxorubicine (adriamycine) ou épipodophyllotoxines (tel l'étoposide (VP-16) ou le téniposide (VM26))], les acridines (tel que le mAMSA) et les anthracendiones (e.g. mitoxantrone) sont des exemples de drogues inhibitrices de topoisomérases II qui stabilisent le complexe de clivage. Plus récemment, une nouvelle classe d'inhibiteurs de topoisomérases II a été développée ; ces inhibiteurs agissent au niveau de l'activité catalytique et non plus en stabilisant le complexe de clivage. La drogue fostriécine en est un exemple (Boritzki *et al.*, 1988). Aujourd'hui ces différentes drogues sont utilisées dans des traitements anti-cancéreux curatifs et palliatifs.

Néanmoins, l'un des problèmes majeurs rencontré dans les traitements anti-cancéreux actuels utilisant les inhibiteurs des topoisomérases est l'émergence d'une résistance aux drogues (Kubo *et al.*, 1995). Ces résistances sont soit le fait d'une surexpression de pompes permettant l'efflux de drogues à l'extérieur de cellules avant qu'elles n'atteignent leur cible (e.g ; P-glycoprotéine, protéine associée à la multirésistance aux drogues (MRP)), soit le fait de changement du taux d'expression de la topoisomérase II α (Deffie *et al.*, 1989; Fry *et al.*, 1991), soit des deux (pour revue, voir Isaacs *et al.*, 1998).

L'un des aspects de la présente invention est donc de comprendre les mécanismes de régulation de l'expression du gène de la topoisomérase II α , afin de développer une alternative au phénomène de résistance aux drogues observé pour certains cancers, et ce, dans l'optique d'améliorer le traitement préventif et curatif des cancers.

Il existe deux types de topoisomérases de type II qui diffèrent dans leur profil d'expression ; la topoisomérase II α (Top II α) (170 kD), essentiellement localisée dans le nucléoplasme au niveau du centromère des chromosomes mitotiques, intervient dans les processus biologiques fondamentaux que sont la réplication, la condensation des chromosomes et la transcription. La topoisomérase II β (Top II β) (180 kD) est semble-t-il plutôt impliquée dans la transcription des ARN ribosomiques étant donné la localisation nucléolaire de cette enzyme. Les deux topoisomérases de type II humaines sont localisées sur deux chromosomes différents (17q21-22 pour la topoisomérase II α et 3p24 pour la topoisomérase II β) (Tsai-Plugfelder *et al.*, 1988 ; Drake *et al.*, 1989 ; Chung *et al.*, 1989 ; Jenkins *et al.*, 1992 ; Austin *et al.*, 1993).

Contrairement à la topoisomérase II β dont l'expression se caractérise par une relative constance, la topoisomérase II α présente une variation d'expression en fonction de l'état de prolifération des cellules et de leur position dans le cycle cellulaire. L'expression de l'ARN messager (ARNm) est plus élevée dans les cellules en prolifération que dans les cellules arrêtées en confluence. L'expression de la topoisomérase II α augmente au cours de la phase S du cycle cellulaire pour atteindre un maximum en fin de phase G2/M (Goswami *et al.*, 1996), le niveau d'ARN messager étant dix fois plus élevé en fin de phase S que pendant la phase G1. Egalement, il semble exister un couplage entre la synthèse et la dégradation de la topoisomérase II α et la condensation/décondensation chromosomique (Heck *et al.*, 1988).

Les connaissances actuelles concernant la régulation du gène de la topoisomérase II α restent somme toute assez sommaires. Récemment, une région promotrice d'environ 650 paires de bases a

été décrite par Hochhauser *et al.* (1992), elle présente toutes les caractéristiques d'un gène domestique, absence de boîte TATA et richesse modérée en sites GC (présence notamment d'une boîte Sp1 pouvant remplacer la boîte TATA) en sont deux exemples. La
5 présence de 5 boîtes CCAAT inversées ou ICB (Inverted CCAAT box) est une autre particularité de ce type de promoteur.

Des facteurs de transcription interagissant avec le promoteur du gène de la topoisomérase II α humaine ont été décrits ; on peut citer c-myb (Brandt *et al.*, 1997), p53 (Sandri *et al.*,
10 1996), ATF (Lim *et al.*, 1998), Sp1 et Sp3 (Kubo *et al.*, 1995). Quoiqu'il en soit, en dehors de NF-Y (également appelé CBF, ACF et CP1, références dans Isaacs *et al.*, 1996) les facteurs de transcription agissant sur les séquences ICB du promoteur du gène de la topoisomérase II α humaine ne sont pas encore tous identifiés
15 et caractérisés ; Herzog et Zwellung (1997) ont cependant mis en évidence deux protéines d'un poids moléculaire apparent de 90 kD et de 140 kD qui lient respectivement ICB1 à ICB4 et ICB5. Isaacs et ses collaborateurs (1996) ont proposé que le NFY ainsi qu'une autre protéine non identifiée reconnaissent une boîte ICB de la
20 région promotrice du gène de la topoisomérase II α ; ils ont également montré que les mutations de ICB2 abrogeaient complètement la diminution de l'activité promotrice normalement observée dans des cellules arrêtées à confluence (Isaac *et al.*, 1996). Ils ont identifié NFY comme un composant d'un complexe induit
25 par la prolifération et qui se lie *in vitro* à la séquence ICB2 du promoteur du gène de la topoisomérase II α humaine, bien que NF-Y soit toujours détectable dans les cellules arrêtées à confluence (Isaac *et al.*, 1996). Ils ont proposé que ICB2 agisse comme un régulateur négatif du promoteur du gène de la topoisomérase II α
30 des cellules arrêtées à confluence et que cette répression puisse

être supprimée dans les cellules prolifératives. La boîte ICB2 du promoteur du gène de la topoisomérase II α joue donc un rôle primordial dans l'arrêt du processus prolifératif normal lorsque les cellules arrivent à confluence.

- 5 Des facteurs de transcription se liant à la séquence ICB ainsi que la séquence ICB elle-même constituent donc des cibles moléculaires pour contrôler le taux d'expression de la topoisomérase II α . En intervenant sur ces facteurs, il est possible d'envisager de contrôler l'expression du gène de la topoisomérase
10 II α et par voie de conséquence la prolifération cellulaire.

La présente invention a pour objet la mise en évidence de nouveaux facteurs de transcription se liant la boîte ICB impliquée dans le contrôle de la prolifération cellulaire.

- Une technique récente appelée système « simple-hybride »
15 qui permet d'isoler des clones ADNc codant pour des protéines de liaison à l'ADN spécifique de certaines séquences a été utilisée. Ce système présente un double avantage car il est capable non seulement de mettre à jour des interactions ADN-protéine *in vivo* chez la levure mais aussi de donner directement accès aux ADN
20 complémentaires (ADNc) codant les protéines candidates ayant une activité de facteur transcription. Le système repose principalement sur la construction d'une souche de levure test selon le principe mis au point par Wang et Reed (1993). Cette souche de levure permet le criblage de banques d'ADNc en mettant en évidence
25 l'interaction ADN-protéine *in vivo* par le biais de l'activation d'un gène rapporteur intégré au génome de la levure test.

- La présente invention a donc pour objet un polypeptide isolé dénommé ICBP90 (inverted CCAAT box binding protein) de
30 séquence d'acides aminés SEQ ID N°2. Cette séquence comprend :

- a) un domaine « ubiquitine » comprenant la séquence d'acides aminés 1 à 75 de la séquence SEQ ID N°2 ;
- 5 b) un domaine « doigt de zinc » de type C4HC3 comprenant la séquence d'acides aminés 310 à 366 de la séquence SEQ ID N°2 et un domaine "doigt de zinc" de type C3HC4 comprenant la séquence d'acides aminés 724 à 763 de la séquence ID n° 2;
- 10 c) un domaine « leucine zipper » putatif comprenant la séquence d'acides aminés 58 à 80 de la séquence SEQ ID N°2 ;
- d) deux domaines de localisation nucléaire potentiels comprenant les séquences d'acides aminés 581 à 600 et 648 à 670 de la séquence SEQ ID N°2 ;
- 15 e) un site de phosphorylation par une tyrosine kinase comprenant la séquence d'acides aminés 452 à 458 de la séquence SEQ ID N°2 ;
- 20 f) des sites de phosphorylation par une protéine kinase cAMP/cGMP dépendante comprenant les séquences d'acides aminés 246 à 249, 295 à 298 et 648 à 651 de la séquence SEQ ID N°2 ;
- g) des sites de phosphorylation par une caséine kinase II comprenant la séquence d'acides aminés 23 à 26, 57 à 60, 91 à 94, 109 à 112, 165 à 168, 265 à 268, 354 à 357 et 669 à 672 de la séquence SEQ ID N°2 ;
- 25 h) des sites de phosphorylation par une protéine kinase C comprenant la séquence d'acides aminés 82 à 84, 104 à 106, 160 à 162, 173 à 175, 251 à 253, 301 à 303, 380 à 382, 393 à 395, 504 à 506, 529 à 531, 625 à 627 et 639 à 641 de la séquence SEQ ID N°2 .

La présente invention porte également sur un polypeptide isolé caractérisé en ce qu'il comprend un polypeptide choisi parmi :

- a) un polypeptide de séquence SEQ ID N°2, SEQ ID N°4, SEQ ID N°6 ou SEQ ID N°8 ;
- 5 b) un polypeptide variant de polypeptide de séquences d'acides aminés défini en a) ;
- c) un polypeptide homologue au polypeptide défini en a) ou b) et comportant au moins 80 % d'homologie, de préférence 90 % avec ledit polypeptide de a) ;
- 10 d) un fragment d'au moins 5 acides aminés consécutifs d'un polypeptide défini en a), b) ou c) ;
- e) un fragment biologiquement actif d'un polypeptide défini en a), b) ou c).

Il doit être compris que l'invention concerne les polypeptides
15 obtenus par purification à partir de sources naturelles, ou bien obtenues par recombinaison génétique, ou encore par synthèse chimique et pouvant alors comporter des acides aminés non naturels.

Dans la présente description, on utilisera le terme
20 polypeptide pour désigner également une protéine ou un peptide.

On entendra par polypeptide variant l'ensemble des polypeptides mutés pouvant exister naturellement, en particulier chez l'être humain, et qui correspondent notamment à des troncatures, substitutions, délétions et/ou additions de résidus
25 d'acides-amino. Les polypeptides homologues selon l'invention conserve au moins un domaine choisi parmi le domaine de liaison à l'ADN et/ou le domaine d'interaction avec une autre protéine.

Par polypeptide homologue, on entendra désigner les polypeptides présentant, par rapport au polypeptide naturel
30 ICBP90, certaines modifications comme en particulier une délétion,

addition ou substitution d'au moins un acide aminé, une troncature, un allongement et/ou une fusion chimérique. Parmi les polypeptides homologues, on préfère ceux dont la séquence d'acides aminés présente au moins 80 % d'homologie, de préférence 90 %, de manière préférée 95 %, et de manière encore préférée 97 % d'homologie avec les séquences d'acides aminés des polypeptides selon l'invention. Dans le cas d'une substitution, un ou plusieurs acides aminés consécutifs ou non consécutifs, sont remplacés par des acides aminés « équivalents ». L'expression acide aminé « équivalent » vise ici à désigner tout acide aminé susceptible d'être substitué à l'un des acides aminés de la structure de base sans cependant modifier les caractéristiques ou propriétés fonctionnelles essentielles, comme leurs activités biologiques, des polypeptides correspondants telles que l'induction *in vivo* d'anticorps capables de reconnaître le polypeptide dont la séquence d'acides aminés est comprise dans la séquence d'acides aminés SEQ ID N°2, ou l'un de ses fragments ci-dessus définis et notamment la séquence d'acides aminés SEQ ID N°4, SEQ ID N°6 et SEQ ID N°8 . Ces acides aminés équivalents peuvent être déterminés soit en s'appuyant sur leur homologie de structure avec les acides aminés auxquels ils se substituent, soit sur les résultats des essais d'activité biologique croisée auxquels les différents polypeptides sont susceptibles de donner lieu. A titre d'exemple, on mentionnera les possibilités de substitutions susceptibles d'être effectuées sans qu'il en résulte une modification approfondie des activités biologiques des polypeptides modifiés correspondants, les remplacements, par exemple, de la leucine par la valine ou l'isoleucine, de l'acide aspartique par l'acide glutamique, de la glutamine par l'asparagine, de l'arginine par la lysine etc., les substitutions inverses étant naturellement envisageables dans les mêmes conditions.

Par fragment biologiquement actif, on entendra désigner en particulier un fragment de séquence d'acides aminés de polypeptide selon l'invention présentant au moins une des caractéristiques ou propriétés fonctionnelles des polypeptides selon l'invention, notamment en ce que : (i) il est capable d'être reconnu par un anticorps spécifique d'un polypeptide selon l'invention ; (ii) il présente au moins l'un des domaines ou régions tels que définis ci-après ; (iii) il est capable de se lier à l'ADN et notamment aux boîtes CCAATT et/ou CCAAT inversée ; (iv) il est capable de moduler le taux d'expression du gène de la topoisomérase II α ; (v) il est capable de moduler la prolifération cellulaire.

Par fragment de polypeptide, on entend désigner un polypeptide comportant au minimum 5 acides aminés, de préférence 7 acides aminés, de manière préférée 10 et de manière encore préférée 15 acides aminés. Les fragments de polypeptide selon l'invention obtenus par clivage dudit polypeptide par une enzyme protéolytique, par un réactif chimique, ou encore en plaçant ledit polypeptide dans un environnement très acide font également partie de l'invention.

Le polypeptide selon l'invention peut également s'associer à d'autres polypeptides par des interactions protéine-protéine. On entend désigner par interactions protéine-protéine, des associations mettant directement en contact au moins deux protéines. Ainsi, le polypeptide de l'invention peut se dimériser pour former des homodimères ou des hétérodimères, ou s'associer sous la forme d'homomultimères ou d'hétéromultimères. Le polypeptide selon l'invention peut également interagir avec un autre polypeptide pour exercer son action ; ainsi, le polypeptide selon l'invention peut posséder, en plus de son domaine de liaison à l'ADN, un domaine d'action sur la transcription qui exerce son action via des

interactions protéine-protéine avec d'autres composants protéique de la machinerie transcriptionnelle. On entend désigner par composant protéique de la machinerie transcriptionnelle tous les facteurs de transcription nécessaires à la réalisation et à la
5 régulation de la réaction de transcription.

Le polypeptide selon l'invention est caractérisé en ce qu'il est capable de se lier à une séquence d'ADN et en ce qu'il est comporte au moins un domaine de fixation à l'ADN sélectionné dans le groupe composé d'un domaine « doigt de zinc » (zinc-finger) et d'un
10 domaine « leucine zipper » ; la séquence d'ADN sur laquelle se lie ledit polypeptide est une boîte CCAAT, de préférence une boîte CCAAT inversée (inverted CCAAT box : ICB).

On entend désigner par liaison à une séquence d'ADN, une interaction spécifique entre le polypeptide de l'invention et une
15 séquence d'ADN au moyen d'une série de liaisons faibles contractées entre les acides aminés de la protéine et les bases. Le polypeptide selon l'invention possède au moins un domaine de liaison à l'ADN qui contient au moins un des motifs protéiques connus susceptibles d'interagir avec l'ADN, c'est-à-dire la structure
20 en doigt de gant à laquelle est associée un atome de zinc (« zinc-finger »), la structure hélice-tour-hélice, la structure hélice-boucle-hélice et la fermeture éclair à leucines (« leucine-zipper »).

Par motif en doigt de gant (« zinc-finger »), on entend désigner une séquence d'une vingtaine d'acides aminés ayant dans l'espace
25 une forme de doigt de gant. Il en existe deux types : ceux qui contiennent quatre cystéines (C4) et ceux qui contiennent deux cystéines et deux histidines (C2H2). Ces acides aminés définissent la nature du doigt de gant et sont situés à sa base et un ion Zn^{++} est situé au centre du carré formé par ces quatre acides aminés. Le

polypeptide selon l'invention possède potentiellement deux motifs de type C4.

Par motif de type « leucine zipper », on entend désigner des motifs appartenant à des facteurs de transcription dimérique qui sont soit des homodimères, soit des hétérodimères. Le monomère est constitué d'une séquence à caractère basique qui interagit de manière spécifique avec l'ADN et d'un domaine hydrophobe en hélice α qui interagit avec le domaine homologue de l'autre chaîne. Dans ce domaine se trouve une leucine tous les 7 aminoacides, c'est-à-dire à chaque tour d'hélice. Toutes ces leucines sont alignées et l'interaction se fait à leur niveau entre les deux monomères. Le polypeptide selon l'invention possède potentiellement un motif de type « leucine zipper ».

L'invention concerne également un polynucléotide isolé caractérisé en ce qu'il code pour un polypeptide de séquence SEQ ID N°1 tel que défini précédemment. De manière préférée, le polynucléotide selon l'invention possède la séquence SEQ ID N°1.

L'invention concerne également le polynucléotide isolé caractérisé en ce qu'il comprend un polynucléotide choisi parmi :

- a) un polynucléotide de séquence SEQ ID N°1, SEQ ID N°3, SEQ ID N°5 ou SEQ ID N°7 ou dont la séquence est celle de l'ARN correspondant à la séquence SEQ ID N°1, SEQ ID N°3, SEQ ID N°5 ou SEQ ID N°7 ;
- b) un polynucléotide dont la séquence est complémentaire de la séquence d'un polynucléotide défini en a),
- c) un polynucléotide dont la séquence comporte au moins 80% d'homologie avec un polynucléotide défini en a) ou b),
- d) un polynucléotide hybridant dans des conditions de forte stringence avec une séquence de polynucléotide défini en a), b) ou c),

e) un fragment d'au moins 15 nucléotides consécutifs, de préférence 21 nucléotides consécutifs, et de manière préférée 30 nucléotides consécutifs d'un polynucléotide défini en a), b), c) ou d) à l'exception de l'EST humain AI 084 125, à l'exception de la séquence correspondant à la séquence SEQ ID N° 944 publiée le 5 août 1999 dans la demande de brevet WO 99 38972 et à l'exception des séquences SEQ ID N°9, N°10 et N°11 correspondant respectivement aux EST humains N° AI 083 773, N° AA 811 055, N° AA 488 755, N° AA 129 794 et N° AA 354 253 présentes dans les bases de données d'EST humains (« human dbest»).

Dans la présente description, on entendra désigner par polynucléotide, oligonucléotide, séquence de polynucléotide, séquence nucléotidique ou acide nucléique un fragment d'ADN, aussi bien un ADN double brin, un ADN simple brin que des produits de transcription desdits ADNs, et/ou un fragment d'ARN, lesdits fragments naturels isolés, ou de synthèse, comportant ou non des nucléotides non naturels, désignant un enchaînement précis de nucléotides, modifiés ou non, permettant de définir un fragment ou une région d'un acide nucléique.

Par polynucléotide de séquence complémentaire, on entend désigner tout ADN dont les nucléotides sont complémentaires de ceux de la SEQ ID N° 1, SEQ ID N°3, SEQ ID N°5, SEQ ID N°7 ou d'une partie de la SEQ ID N° 1, SEQ ID N°3, SEQ ID N°5, SEQ ID N°7 et dont l'orientation est inversée.

Par pourcentage d'homologie au sens de la présente invention, on entend un pourcentage d'identité entre les bases de deux polynucléotides, ce pourcentage étant purement statistique et les différences entre les deux polynucléotides étant réparties au

hasard et sur toute leur longueur. Selon l'invention, les polynucléotides de séquence nucléique homologue présentent un taux d'homologie d'au moins 80 %, de préférence 90 %, de manière préférée 95 %, de manière encore préférée 97 %.

- 5 Une hybridation dans des conditions de forte stringence signifie que les conditions de température et de force ionique sont choisies de telle manière qu'elles permettent le maintien de l'hybridation entre deux fragments d'ADN complémentaires. A titre illustratif, des conditions de forte stringence de l'étape
10 d'hybridation aux fins de définir les fragments polynucléotidiques décrits ci-dessus, sont avantageusement les suivantes :

L'hybridation ADN-ADN ou ADN-ARN est réalisée en deux étapes: (1) préhybridation à 42°C pendant 3 heures en tampon phosphate (20 mM, pH 7,5) contenant 5 x SSC (1 x SSC correspond
15 à une solution 0,15 M NaCl + 0,015 M citrate de sodium), 50 % de formamide, 7 % de sodium dodécyl sulfate (SDS), 10 x Denhard's, 5 % de dextran sulfate et 1 % d'ADN de sperme de saumon ; (2) hybridation proprement dite pendant 20 heures à une température dépendant de la taille de la sonde (i.e. : 42°C, pour une sonde de
20 taille > 100 nucléotides) suivie de 2 lavages de 20 minutes à 20°C en 2 x SSC + 2 % SDS, 1 lavage de 20 minutes à 20°C en 0,1 x SSC + 0,1 % SDS. Le dernier lavage est pratiqué en 0,1 x SSC + 0,1 % SDS pendant 30 minutes à 60°C pour une sonde de taille > 100 nucléotides. Les conditions d'hybridation de forte stringence
25 décrites ci-avant pour un polynucléotide de taille définie, seront adaptées par l'homme du métier pour des oligonucléotides de taille plus grande ou plus petite, selon l'enseignement de Sambrook *et al.*, 1989.

Avantageusement, un fragment nucléotidique répondant à la
30 définition précédente aura au moins 15 nucléotides consécutifs, de

préférence au moins 21 nucléotides, et encore plus préférentiellement au moins 30 nucléotides consécutifs de la séquence dont il est issu.

Par EST (« expressed sequence tag »), on entend désigner des
5 séquences exprimées, caractérisées dans une banque d'ADN complémentaire (ADNc) et utilisées comme balise cartographique de l'ADN génomique.

Selon un mode de réalisation de l'invention, le polynucléotide selon l'invention se caractérise en ce qu'il est marqué directement
10 ou indirectement par un composé radioactif ou un composé non radioactif. Utilisation d'un polynucléotide selon l'invention en tant qu'amorce pour l'amplification ou la polymérisation de séquences nucléiques ; l'invention porte également sur l'utilisation d'un polynucléotide selon l'invention en tant que sonde pour la détection
15 de séquences nucléiques. Selon l'invention, les fragments de polynucléotides pouvant être utilisés comme sonde ou comme amorce dans des procédés de détection, d'identification, de dosage ou d'amplification de séquence nucléique, présenteront une taille minimale de 9 bases, de préférence de 18 bases, et de manière plus
20 préférée 36 bases. Enfin, l'invention porte sur l'utilisation d'un polynucléotide selon l'invention en tant que séquence d'acide nucléique sens ou antisens pour contrôler l'expression du produit protéique correspondant.

Les séquences de polynucléotides selon l'invention non
25 marquées peuvent être utilisées directement comme sonde, amorce ou oligonucléotide ; cependant les séquences utilisées sont généralement marquées pour obtenir des séquences utilisables pour de nombreuses applications. Le marquage des amorces, des sondes, des oligonucléotides selon l'invention est réalisé par des
30 éléments radioactifs ou par des molécules non radioactives ; parmi

les isotopes radioactifs utilisés, on peut citer le ^{32}P , le ^{33}P , le ^{35}S , le ^3H ou le ^{125}I . Les entités non radioactives sont sélectionnées parmi les ligands tels la biotine, l'avidine, la streptavidine, la dioxygénine, les haptènes, les colorants, les agents luminescents tels que les agents radioluminescents, chémiluminescents, bioluminescents, fluorescents, phosphorescents.

Les polynucléotides selon l'invention peuvent ainsi être utilisés comme amorce et/ou sonde dans des procédés mettant en oeuvre notamment la technique PCR (réaction en chaîne à la polymérase)(Erich, 1989 ; Innis *et al.*, 1990, et Rolfs *et al.*, 1991). Cette technique nécessite le choix de paires d'amorces oligonucléotidiques encadrant le fragment qui doit être amplifié. On peut, par exemple, se référer à la technique décrite dans le brevet américain U.S. N° 4 683 202. Les fragments amplifiés peuvent être identifiés, par exemple après une électrophorèse en gel d'agarose ou de polyacrylamide, ou après une technique chromatographique comme la filtration sur gel ou la chromatographie échangeuse d'ions. La spécificité de l'amplification peut être contrôlée par hybridation moléculaire en utilisant comme sonde les séquences nucléotidiques de polynucléotides de l'invention, des plasmides contenant ces séquences ou leurs produits d'amplification. Les fragments nucléotidiques amplifiés peuvent être utilisés comme réactifs dans des réactions d'hybridation afin de mettre en évidence la présence, dans un échantillon biologique, d'un acide nucléique cible de séquence complémentaire à celle desdits fragments nucléotidiques amplifiés.

L'invention vise également les fragments nucléotidiques susceptibles d'être obtenus par amplification à l'aide d'amorces selon l'invention.

D'autres techniques d'amplification de l'acide nucléique cible peuvent être avantageusement employées comme alternative à la PCR (PCR-like) à l'aide de couple d'amorces de séquences nucléotidiques selon l'invention. Par PCR-like on entendra désigner
5 toutes les méthodes mettant en oeuvre des reproductions directes ou indirectes des séquences d'acides nucléiques, ou bien dans lesquelles les systèmes de marquage ont été amplifiés, ces techniques sont bien entendu connues, en général il s'agit de l'amplification de l'ADN par une polymérase ; lorsque l'échantillon
10 d'origine est un ARN il convient préalablement d'effectuer une transcription inverse. Il existe actuellement de très nombreux procédés permettant cette amplification, comme par exemple la technique SDA (Strand Displacement Amplification) ou technique d'amplification à déplacement de brin (Walker *et al.*, 1992), la
15 technique TAS (Transcription-based Amplification System) décrite par Kwoh *et al.* en 1989, la technique 3SR (Self-Sustained Sequence Replication) décrite par Guatelli *et al.* en 1990, la technique NASBA (Nucleic Acid Sequence Based Amplification) décrite par Kievitis *et al.* en 1991, la technique TMA (Transcription
20 Mediated Amplification), la technique LCR (Ligase Chain Reaction) décrite par Landegren *et al.* en 1988 et perfectionnée par Barany *et al.* en 1991, qui emploie une ligase thermostable, la technique de RCR (Repair Chain Reaction) décrite par Segev en 1992, la technique CPR (Cycling Probe Reaction) décrite par Duck *et al.* en
25 1990, la technique d'amplification à la Q-bêta-réplique décrite par Miele *et al.* en 1983 et perfectionnée notamment par Chu *et al.* en 1986 et Lizardi *et al.* en 1988, puis par Burg *et al.* ainsi que par Stone *et al.* en 1996.

Dans le cas où le polynucléotide cible à détecter est un ARN,
30 par exemple un ARNm, on utilisera avantageusement,

préalablement à la mise en oeuvre d'une réaction d'amplification à l'aide des amorces selon l'invention ou à la mise en oeuvre d'un procédé de détection à l'aide des sondes de l'invention, une enzyme de type transcriptase inverse afin d'obtenir un ADNc à partir de
5 l'ARN contenu dans l'échantillon biologique. L'ADNc obtenu servira alors de cible pour les amorces ou les sondes mises en oeuvre dans le procédé d'amplification ou de détection selon l'invention.

Les sondes nucléotidiques selon l'invention hybrident spécifiquement avec une molécule d'ADN ou d'ARN de
10 polynucléotide selon l'invention, plus particulièrement avec la séquence SEQ ID N° 1 codant pour le polypeptide ICBP90, dans des conditions d'hybridation de forte stringence telles que données sous forme d'exemple précédemment.

La technique d'hybridation peut être réalisée de manières
15 diverses (Matthews *et al.*, 1988). La méthode la plus générale consiste à immobiliser l'acide nucléique extrait des cellules de différents tissus ou de cellules en culture sur un support (tel que la nitrocellulose, le nylon, le polystyrène) et à incubé, dans des conditions bien définies, l'acide nucléique cible immobilisé avec la
20 sonde. Après l'hybridation, l'excès de sonde est éliminé et les molécules hybrides formées sont détectées par la méthode appropriée (mesure de la radioactivité, de la fluorescence ou de l'activité enzymatique liée à la sonde).

Selon un autre mode de mise en oeuvre des sondes
25 nucléiques selon l'invention, ces dernières peuvent être utilisées comme sonde de capture. Dans ce cas, une sonde, dite « sonde de capture », est immobilisée sur un support et sert à capturer par hybridation spécifique l'acide nucléique cible obtenu à partir de l'échantillon biologique à tester et l'acide nucléique cible est ensuite

détecté grâce à une seconde sonde, dite « sonde de détection », marquée par un élément facilement détectable.

Dans un mode préféré de réalisation, l'invention comprend l'utilisation d'un oligonucléotide sens ou antisens pour contrôler l'expression du produit protéique correspondant. Parmi les fragments d'acides nucléiques intéressants, il faut citer en particulier les oligonucléotides anti-sens, c'est-à-dire dont la structure assure, par hybridation avec la séquence cible, une inhibition de l'expression du produit correspondant. Il faut également citer les oligonucléotides sens qui, par interaction avec des protéines impliquées dans la régulation de l'expression du produit correspondant, induiront soit une inhibition, soit une activation de cette expression. Les oligonucléotides selon l'invention présentent une taille minimale de 9 bases, de préférence de 18 bases, et de manière plus préférée 36 bases.

L'invention concerne un vecteur recombinant de clonage d'un polynucléotide selon l'invention et/ou d'expression d'un polypeptide selon l'invention caractérisé en ce qu'il contient un polynucléotide selon l'invention tel que précédemment décrit. Le vecteur selon l'invention est caractérisé en ce qu'il comporte les éléments permettant l'expression éventuellement la sécrétion desdites séquences dans une cellule hôte. Ces vecteurs sont utiles pour transformer des cellules hôtes afin de cloner ou d'exprimer les séquences nucléotidiques de l'invention. Des vecteurs particuliers sont par exemple des vecteurs d'origine virale ou plasmidique. Parmi ces vecteurs, on préfère ceux de la série pGEX (Pharmacia) pour l'expression dans les bactéries ou pSG5 (Stratagene, La Jolla, CA USA) pour l'expression en système eucaryote.

Selon un mode particulier de réalisation, le vecteur selon l'invention comporte des éléments de contrôle de l'expression des

polypeptides ; ces éléments de contrôle sont choisis de préférence parmi (i) la séquence promotrice du gène ICBP90 selon l'invention qui correspond à la séquence SEQ ID N°12 ; (ii) un polynucléotide dont la séquence est complémentaire à la séquence
5 SEQ ID N° 12 ; (iii) un polynucléotide dont la séquence comporte au moins 80% d'identité avec un polynucléotide défini en (i) ou (ii) ; (iv) un polynucléotide hybridant dans des conditions de forte stringence avec la séquence de polynucléotide définie en (i), (ii), (iii). Les outils informatiques à la disposition de l'homme du métier lui
10 permettent aisément d'identifier les boîtes régulatrices promotrices nécessaires et suffisantes au contrôle de l'expression génique, notamment les boîtes TATA, CCAAT, GC, ainsi que les séquences régulatrices stimulatrices (« enhancer ») ou inhibitrices (« silencers ») qui contrôlent en CIS l'expression des gènes selon l'invention.

15 Il est également dans l'étendue de l'invention d'utiliser les éléments ci-dessus définis et choisis parmi la séquence SEQ ID N°12 pour contrôler l'expression de polypeptides hétérologues autres que ceux de l'invention et notamment pour diriger l'expression de polypeptides hétérologues dans les types cellulaires
20 dans lesquels les polypeptides selon l'invention s'expriment normalement.

L'invention comprend en outre les cellules hôtes, notamment les cellules eucaryotes et procaryotes, caractérisées en ce qu'elles sont transformées par les vecteurs selon l'invention. De
25 préférence, les cellules hôtes sont transformées dans des conditions permettant l'expression d'un polypeptide recombinant selon l'invention. L'hôte cellulaire peut être choisi parmi les cellules bactériennes (Olins et Lee, 1993), mais également les cellules de levure (Buckholz, 1993), de même que les cellules animales, en
30 particulier les cultures de cellules de mammifères (Edwards et

Aruffo, 1993), mais également les cellules d'insectes dans lesquelles on peut utiliser des procédés mettant en oeuvre des baculovirus par exemple (Luckow, 1993). Ces cellules peuvent être obtenues par l'introduction dans des cellules hôtes d'une séquence nucléotidique
5 insérée dans un vecteur tel que défini ci-dessus, puis la mise en culture desdites cellules dans des conditions permettant la réplication et/ou l'expression de la séquence nucléotidique transfectée.

L'invention concerne également une méthode de préparation
10 d'un polypeptide caractérisé en ce qu'il met en œuvre un vecteur selon l'invention. Plus particulièrement, l'invention porte sur une méthode de préparation d'un polypeptide recombinant caractérisé en ce que l'on cultive des cellules transformées selon l'invention dans des conditions permettant l'expression dudit polypeptide
15 recombinant et que l'on récupère ledit polypeptide recombinant.

Le polypeptide selon l'invention est susceptible d'être obtenu selon un procédé de l'invention et selon les techniques de production de polypeptides recombinants connues de l'homme du métier. La présente invention concerne donc le polypeptide
20 recombinant susceptible d'être obtenu par la méthode ci-dessus présentée. Dans ce cas, la séquence d'acide nucléique utilisée est placée sous le contrôle de signaux permettant son expression dans un hôte cellulaire. Un système efficace de production d'un polypeptide recombinant nécessite de disposer d'un vecteur, par
25 exemple d'origine plasmidique ou virale, et d'une cellule hôte compatible. Le vecteur doit comporter un promoteur, des signaux d'initiation et de terminaison de la traduction, ainsi que des régions appropriées de régulation de la transcription. Il doit pouvoir être
30 posséder des signaux particuliers spécifiant la sécrétion du

polypeptide traduit. Ces différents signaux de contrôle sont choisis en fonction de l'hôte cellulaire utilisé. A cet effet, les séquences d'acide nucléique selon l'invention peuvent être insérées dans des vecteurs à réplication autonome au sein de l'hôte choisi, ou des
5 vecteurs intégratifs de l'hôte choisi. De tels vecteurs seront préparés selon les méthodes couramment utilisées par l'homme du métier, et les clones en résultant peuvent être introduits dans un hôte approprié par des méthodes standards telles par exemple la transfection par précipitation au phosphate de calcium, la
10 lipofection, l'électroporation, le choc thermique.

Les polypeptides recombinants obtenus comme indiqué ci-dessus, peuvent aussi bien se présenter sous forme glycosylée que non glycosylée et peuvent présenter ou non la structure tertiaire naturelle.

15 Les polypeptides obtenus par synthèse chimique et pouvant comporter des acides aminés non naturels correspondant auxdits polypeptides recombinants, sont également compris dans l'invention. Les peptides selon l'invention peuvent également être préparés par les techniques classiques, dans le domaine de la
20 synthèse des peptides. Cette synthèse peut être réalisée en solution homogène ou en phase solide.

Les procédés de purification de polypeptide recombinant utilisés sont connus de l'homme du métier. Le polypeptide recombinant peut être purifié à partir de lysats et extraits
25 cellulaires, du surnageant du milieu de culture, par des méthodes utilisées individuellement ou en combinaison, telles que le fractionnement, les méthodes de chromatographie, les techniques d'immuno-affinité à l'aide d'anticorps mono ou polyclonaux spécifiques, etc.

Une variante préférée consiste à produire un polypeptide recombinant fusionné à une protéine « porteuse » (protéine chimère). L'avantage de ce système est qu'il permet une stabilisation et une diminution de la protéolyse du produit recombinant, une augmentation de la solubilité au cours de la renaturation *in vitro* et/ou une simplification de la purification lorsque le partenaire de fusion possède une affinité pour un ligand spécifique.

L'invention concerne également un anticorps monoclonal ou polyclonal et ses fragments, caractérisés en ce qu'ils lient spécifiquement un polypeptide selon l'invention. Les anticorps chimériques, les anticorps humanisés et les anticorps simple chaîne font également partie de l'invention. Les fragments d'anticorps selon l'invention sont de préférence des fragments Fab ou F(ab')₂.

Les polypeptides selon l'invention permettent de préparer des anticorps monoclonaux ou polyclonaux. Les anticorps monoclonaux pourront avantageusement être préparés à partir d'hybridomes selon la technique décrite par Kohler et Milstein en 1975. Les inventeurs ont employé cette technique pour obtenir un hybridome produisant un nouvel anticorps monoclonal hautement spécifique d'un épitope de la protéine ICBP90.

Les anticorps polyclonaux pourront être préparés, par exemple par immunisation d'un animal, en particulier une souris, avec un polypeptide selon l'invention associé à un adjuvant de la réponse immunitaire, puis purification des anticorps spécifiques contenus dans le sérum des animaux immunisés sur une colonne d'affinité sur laquelle a préalablement été fixé le polypeptide ayant servi d'antigène. Les anticorps polyclonaux selon l'invention peuvent aussi être préparés par purification sur une colonne d'affinité, sur

laquelle a préalablement été immobilisé un polypeptide selon l'invention.

L'invention porte également sur un anticorps monoclonal spécifique de la protéine ICBP90 humaine et capable d'inhiber
5 l'interaction entre ICBP90 et la séquence d'ADN sur laquelle se lie spécifiquement la protéine ICBP90. Selon un autre mode de réalisation, l'anticorps monoclonal selon l'invention et spécifique de la protéine ICBP90 humaine est capable d'inhiber l'interaction entre ICBP90 et les protéines avec lesquelles ICBP90 interagit, lesdites
10 protéines étant de préférence ICBP90 elle-même ou des protéines du complexe transcriptionnel. Par protéines du complexe transcriptionnel, on entend désigner toutes les protéines intervenant dans la réaction de la transcription que se soit l'initiation, l'élongation ou la terminaison de la transcription.

15 Les anticorps de l'invention pourront également être marqués de la même manière que décrit précédemment pour les sondes nucléiques de l'invention et de manière préférée avec un marquage de type enzymatique, fluorescent ou radioactif.

Par ailleurs, outre leur utilisation pour la purification des
20 polypeptides, les anticorps de l'invention, en particulier les anticorps monoclonaux, peuvent également être utilisés pour la détection de ces polypeptides dans un échantillon biologique.

Ils constituent ainsi un moyen d'analyse de l'expression de polypeptide selon l'invention, par exemple par immunofluorescence,
25 marquage à l'or, immunoconjugués enzymatiques.

Plus généralement, les anticorps de l'invention peuvent être avantageusement mis en oeuvre dans toute situation où l'expression d'un polypeptide selon l'invention doit être observée, et plus particulièrement en immunocytochimie, en

immunohistochimie ou dans des expériences de « western blotting ».

Ainsi, l'invention concerne une méthode de détection et/ou de dosage d'un polypeptide selon l'invention, dans un échantillon
5 biologique, caractérisé en ce qu'il comprend les étapes suivantes de mise en contact de l'échantillon biologique avec des anticorps selon l'invention puis de mise en évidence du complexe antigène-anticorps formé. Cette méthode peut être utilisée en immunocytochimie pour la localisation cellulaire du polypeptide
10 selon l'invention et en immunohistochimie pour évaluer la prolifération cellulaire.

Entre également dans le cadre de l'invention, un nécessaire pour la détection et/ou le dosage d'un polypeptide selon l'invention dans un échantillon biologique, caractérisé en ce qu'il comprend les
15 éléments suivants : (i) un anticorps monoclonal ou polyclonal tel que décrit précédemment ; (ii) le cas échéant, les réactifs pour la constitution du milieu propice à la réaction immunologique ; (iii) les réactifs permettant la détection des complexes antigène-anticorps produits par la réaction immunologique. Ce kit est notamment utile
20 à la réalisation d'expériences de Western Blotting ; celles-ci permettent d'étudier la régulation de l'expression du polypeptide selon l'invention à partir de tissus ou de cellules. Ce kit est également utile aux expériences d'immunoprécipitation pour mettre en évidence notamment les protéines interagissant avec le
25 polypeptide selon l'invention.

Toute procédure classique peut être mise en oeuvre pour réaliser une telle détection et/ou dosage. A titre d'exemple, une méthode préférée met en jeu des processus immunoenzymatiques selon la technique ELISA, par immunofluorescence, ou radio-
30 immunologique (RIA) ou équivalent.

L'invention comprend également une méthode de détection et/ou de dosage d'acide nucléique selon l'invention, dans un échantillon biologique, caractérisé en ce qu'il comporte les étapes suivantes : (i) d'isolement de l'ADN à partir de l'échantillon
5 biologique à analyser, ou obtention d'un ADNc à partir de l'ARN de l'échantillon biologique ; (ii) d'amplification spécifique de l'ADN codant pour le polypeptide selon l'invention à l'aide d'amorces ; (iii) d'analyse des produits d'amplification.

L'invention comprend en outre un nécessaire pour la
10 détection et/ou le dosage d'un acide nucléique selon l'invention, dans un échantillon biologique, caractérisé en ce qu'il comprend les éléments suivants : (i) un couple d'amorces nucléiques selon l'invention, (ii) les réactifs nécessaires pour effectuer une réaction d'amplification d'ADN, et éventuellement (iii) un composant
15 permettant de vérifier la séquence du fragment amplifié, plus particulièrement une sonde selon l'invention.

L'invention comprend aussi une méthode de détection et/ou de dosage d'acide nucléique selon l'invention, dans un échantillon biologique, caractérisé en ce qu'il comporte les étapes suivantes : (i)
20 de mise en contact d'une sonde selon l'invention avec un échantillon biologique ; (ii) de détection et/ou de dosage de l'hybride formé entre ladite sonde et l'ADN de l'échantillon biologique.

L'invention comprend également un nécessaire pour la détection et/ou le dosage d'acide nucléique selon l'invention, dans
25 un échantillon biologique, caractérisé en ce qu'il comprend les éléments suivants : (i) une sonde selon l'invention, (ii) les réactifs nécessaires à la mise en oeuvre d'une réaction d'hybridation, et le cas échéant, (iii) un couple d'amorces selon l'invention, ainsi que les réactifs nécessaires à une réaction d'amplification de l'ADN.

L'invention concerne particulièrement les procédés selon l'invention et décrits ci-dessus, pour la détection et le diagnostic de prolifération cellulaire, et plus particulièrement de prolifération cellulaire d'origine cancéreuse.

- 5 L'invention concerné également une méthode de criblage de ligands susceptibles d'affecter l'activité transcriptionnelle d'un gène dont le promoteur comporte des boîtes CCAAT et/ou CCAAT inversées susceptibles de lier un polypeptide selon l'invention, ladite méthode étant caractérisée en ce qu'elle comporte les étapes
- 10 suivantes de mise en contact dudit polypeptide et d'un ou plusieurs ligand(s) potentiel(s) en présence de réactifs nécessaires à la mise en œuvre d'une réaction de transcription ou de détection et/ou de mesure de l'activité transcriptionnelle. C'est également un des objets de l'invention de fournir un kit ou un nécessaire pour le
- 15 criblage de ligands susceptibles d'affecter l'activité transcriptionnelle d'un gène dont le promoteur comporte des boîtes CCAAT et/ou CCAAT inversées susceptibles de lier un polypeptide selon l'invention caractérisé en ce qu'il comprend les éléments suivants : (i) un polypeptide selon l'invention ; (ii) un ligand ; (iii) les
- 20 réactifs nécessaires à la mise en œuvre d'une réaction de transcription.

Le polypeptide ICBP90 selon l'invention présente une fonction de récepteur nucléaire. Par récepteur nucléaire, on entend désigner un polypeptide qui possède les propriétés essentielles des

25 récepteurs nucléaires d'hormones. Cette superfamille de gène contient entre autres les récepteurs nucléaires à l'acide rétinoïque (RAR, RXR,...), les récepteurs nucléaires aux hormones stéroïdes (glucocorticoïdes, minéralocorticoïdes, progestérone, androgène, œstrogène), et les récepteurs nucléaires aux hormones

30 thyroïdiennes (hormone T3). C'est donc également un des objets de

la présente invention de fournir un procédé de criblage de ligand susceptible d'affecter la fonction « récepteur nucléaire » du polypeptide selon l'invention. Un tel procédé comporte les étapes de :

- 5 a) mise en contact du polypeptide de l'invention et d'un ou plusieurs ligands potentiels en présence de réactifs nécessaires ;
- b) détection et/ou mesure de l'activité transcriptionnelle d'un gène dont le promoteur comporte des séquences
10 nucléotidiques sur lesquelles sont susceptibles de se lier le polypeptide de l'invention. De préférence, lesdites séquences nucléotidiques sont des boîtes CCAAT et/ou CCAAT inversées (ICB).

Les techniques de détection et/ou de mesure de l'activité
15 transcriptionnelle sont connues de l'homme du métier. Il convient notamment de citer les technologies de Northern Blotting et de RT-PCR qui peuvent être mises en œuvre avec les polynucléotides de l'invention utilisés respectivement comme sonde ou comme amorce.

Par ligand, on entend définir tous les composés susceptibles
20 d'interagir avec le polypeptide selon l'invention pour former un complexe susceptible d'affecter l'activité transcriptionnelle, c'est-à-dire d'augmenter, de diminuer, de moduler ou d'annuler la transcription d'un gène sous le contrôle d'un promoteur contenant une séquence d'ADN à laquelle se lie le polypeptide de l'invention.

25 Un tel ligand est donc susceptible d'avoir une activité agoniste ou antagoniste. Parmi les ligands selon l'invention, il convient de citer les molécules biologiques interagissant avec le polypeptide selon l'invention, ainsi que tous les composés chimiques de synthèse. Parmi les ligands, il convient également de
30 citer l'anticorps selon l'invention, ainsi qu'un oligonucléotide

présentant une identité de séquence avec la séquence nucléotidique CCAAT et/ou CCAAT inversée ; un tel ligand est susceptible de constituer un inhibiteur du polypeptide selon l'invention.

L'invention porte également sur le ligand susceptible d'être
5 obtenu par les procédés de criblage précédents.

On entend définir également par ligand tout composé susceptible de se lier à la séquence d'ADN de liaison du polypeptide selon l'invention. Un tel ligand constitue un inhibiteur compétitif du polypeptide selon l'invention pour la liaison à la séquence
10 d'ADN.

De préférence, l'échantillon biologique selon l'invention dans lequel est réalisé la détection et le dosage est constitué par un fluide corporel, par exemple un sérum humain ou animal, du sang, de la salive, du mucus pulmonaire, ou par des biopsies. Entre
15 également dans la définition d'un échantillon biologique de l'invention le liquide biologique résultant d'un lavage broncho-alvéolaire obtenu également lors des analyses diagnostiques des cancers des voies aériennes profondes.

Selon un autre aspect, l'invention concerne un composé
20 caractérisé en ce qu'il est choisi parmi un anticorps, un polypeptide, un ligand, un polynucléotide, un oligonucléotide ou un vecteur selon l'invention à titre de médicament et notamment en tant que principes actifs de médicament ; ces composés seront préférentiellement sous forme soluble, associés à un véhicule
25 pharmaceutiquement acceptable. Par véhicule pharmaceutiquement acceptable, on entend désigner tout type de véhicule employé habituellement dans la préparation de compositions injectables, c'est-à-dire un diluant, un agent de suspension tel une solution saline isotonique ou tamponnée. De
30 préférence, ces composés seront administrés par voie systémique,

en particulier par voie intraveineuse, par voie intramusculaire, intradermique ou par voie orale. Leurs modes d'administration, posologies et formes galéniques optimaux peuvent être déterminés selon les critères généralement pris en compte dans l'établissement
5 d'un traitement adapté à un patient comme par exemple l'âge ou le poids corporel du patient, la gravité de son état général, la tolérance au traitement et les effets secondaires constatés, etc.

Selon un autre aspect, l'invention concerne un composé caractérisé en ce qu'il est choisi parmi un polypeptide, un
10 polynucléotide, un polynucléotide antisens, un anticorps, un vecteur, une cellule, un ligand selon l'invention à titre de médicament et notamment en tant que principes actifs de médicament ; ces composés seront préférentiellement sous forme soluble, associés à un véhicule pharmaceutiquement acceptable.

15 Par véhicule pharmaceutiquement acceptable, on entend désigner tout type de véhicule employé habituellement dans la préparation de compositions injectables, c'est-à-dire un diluant, un agent de suspension tel une solution saline isotonique ou tamponnée. De préférence, ces composés seront administrés par voie systémique,
20 en particulier par voie intraveineuse, par voie intramusculaire, intradermique ou par voie orale. Leurs modes d'administration, posologies et formes galéniques optimaux peuvent être déterminés selon les critères généralement pris en compte dans l'établissement d'un traitement adapté à un patient comme par exemple l'âge ou le
25 poids corporel du patient, la gravité de son état général, la tolérance au traitement et les effets secondaires constatés, etc. Quand l'agent est un polypeptide, un antagoniste, un ligand, un polynucléotide, par exemple une composition anti-sens, un vecteur, on peut l'introduire dans des tissus ou des cellules hôtes par un certain
30 nombre de façons, incluant l'infection virale, la micro-injection ou

la fusion de vésicules. On peut également utiliser l'injection par jet pour une administration intramusculaire comme décrit par Furth *et al.* (1992). On peut déposer le polynucléotide sur des microparticules d'or, et le délivrer par voie intradermique. à l'aide
5 d'un dispositif de bombardement de particules, ou un « pistolet à gène » comme décrit dans la littérature (voir par exemple Tang *et al.* (1992) où les microprojectiles d'or sont revêtues avec le polynucléotide de l'invention, de préférence le polynucléotide antisens de l'invention, puis bombardée dans les cellules de peau.

10 Le composé selon l'invention est utilisé pour la préparation d'un médicament destiné à moduler, à augmenter ou à diminuer la prolifération cellulaire.

L'invention porte également sur une composition pharmaceutique pour le traitement préventif et curatif du cancer
15 caractérisée en ce qu'elle contient une quantité thérapeutiquement efficace d'un composé selon l'invention et un véhicule pharmaceutiquement acceptable. Selon un mode préféré de réalisation, la composition pharmaceutique est caractérisée en ce qu'elle contient un anticorps selon l'invention en tant qu'agent de
20 ciblage conjugué à au moins un agent sélectionné parmi le groupe des agents antiprolifératifs, antinéoplastiques ou cytotoxiques. Ces agents sont des radioisotopes ou des entités non isotopiques. La conjugaison de l'anticorps de la présente invention à un agent antiprolifératif, antinéoplastique ou cytotoxique peut être utilisé
25 pour arrêter le développement des cancers et pour induire une régression et/ou une élimination de la masse tumorale. De préférence, l'anticorps ou le fragment d'anticorps ainsi conjugué est introduit dans le patient atteint de cancer et délivré aux sites tumoraux par voie orale ou parentérale dans un liquide
30 transporteur pharmaceutiquement acceptable tel qu'une solution

de sel physiologique. Alternativement, une solution ou une suspension d'anticorps ou de fragment d'anticorps conjugué à un agent peut être perfusée directement dans le tissu épithélial malin, cette méthode étant utilisée de préférence dans le cas où le cancer n'est pas métastaté.

Les radioisotopes préférés conjugués aux anticorps monoclonaux employés pour la thérapie sont des radioisotopes émetteurs de rayons gamma et de préférence l'Iode¹³¹, l'Yttrium⁹⁰, l'Or¹⁹⁹, le Palladium¹⁰⁰, le Cuivre⁶⁷, le Bismuth²¹⁷ et l'Antimoine²¹¹.

10 Les radioisotopes émetteurs de rayons beta et alpha peuvent également être utilisés pour la thérapie. Les entités non isotopiques conjuguées aux anticorps monoclonaux employés pour la thérapie sont multiples et variés; on peut citer: (i) les antimétabolites telles les agents anti-folate, le méthotrexate, (ii) les analogues des purines

15 et des pyrimidines (mercaptapurine, fluorouracile, 5-azacytidine), (iii) les antibiotiques, (iv) les lectines (ricine, abrine) et (iv) les toxines bactériennes (toxine diphtérique).

L'anticorps selon l'invention peut également être utilisé en tant qu'agent de ciblage pour cibler des cellules cytotoxiques telles

20 les cellules T humaines, les monocytes ou les cellules NK sur le lieu de la tumeur métastasée ou non. Les cellules cytotoxiques peuvent être attachées à l'anticorps via le récepteur Fc situé à la surface de ces cellules ou via un anticorps intermédiaire présentant une double spécificité par exemple; de tels anticorps bispécifiques pour

25 le ciblage des cellules cancéreuses peuvent être produits en fusionnant une cellule immunitaire produisant l'anticorps de la présente invention ou l'hydridome de la présente invention avec une cellule produisant un anticorps dirigé contre la cellule cytotoxique à cibler. Des anticorps bispécifiques peuvent également

30 être produits par couplage chimique de deux anticorps ayant la

spécificité désirée. L'anticorps selon l'invention permet également de cibler des véhicules de délivrance d'agents antiprolifératifs, antinéoplastiques ou cytotoxiques sur le lieu de la tumeur métastasée ou non. Par véhicules de délivrance on entend désigner
5 les liposomes et les particules virales. Dans certains cas, on pourra prévoir des éléments de ciblage assurant une expression spécifique de certains tissus ou cellules de façon à pouvoir limiter les zones d'expression des polypeptides selon l'invention.

L'invention concerne également un produit comprenant au
10 moins un composé selon l'invention et au moins un agent anticancéreux comme produit de combinaison pour une utilisation simultanée, séparée ou étalée dans le temps en thérapie anticancéreuse.

Enfin, l'invention concerne une composition pour la
15 détection, la localisation et l'imagerie des cancers, comprenant un anticorps selon l'invention, tel que l'anticorps est marqué directement ou indirectement avec un marqueur générateur de signal sélectionné parmi les isotopes radioactifs et les entités non isotopiques tels que définis précédemment. L'invention a également
20 pour objet une méthode de détection, de localisation et d'imagerie du cancer, comprenant (i) les étapes d'injection parentérale chez un être humain d'une composition selon l'invention; (ii) l'accumulation après un temps suffisant au niveau des cellules cancéreuses de l'anticorps marqué puis pénétration de l'anticorps marqué à
25 l'intérieur desdites cellules, sans que ledit anticorps ne se lie de manière substantielle aux cellules normales; et (iii) la détection du signal au moyen d'un détecteur de signal; et (iv) la conversion du signal détecté en une image des cellules cancéreuses.

D'autres caractéristiques et avantages de l'invention apparaissent dans la suite de la description avec les exemples représentés ci-après. Dans ces exemples on se référera aux figures suivantes.

5

Figure 1 : Expression de la protéine ICBP90 dans les cellules HeLa (cellules tumorales) et dans les fibroblastes pulmonaires humains en culture primaire (cellules non tumorales).

La détection de la protéine endogène ICBP90 a été réalisée
10 sur des extraits totaux de protéines de cellules HeLa à confluence (piste 1) ou en prolifération (piste 2) et sur des extraits totaux de protéines de fibroblastes pulmonaires humains en culture primaire à confluence (piste 3) ou en prolifération (piste 4). Après migration sur gel de polyacrylamide 8% en présence de SDS, les protéines
15 sont transférées sur membrane de nitrocellulose par électrotransfert. La révélation est réalisée à l'aide de l'anticorps 1RC1C-10 dilué au 1/4000 (concentration initiale 2 mg/ml) et d'un anticorps secondaire couplé à la phosphatase alcaline et dirigé contre les chaînes lourdes d'anticorps de souris. Dans les pistes
20 correspondant aux extraits de cellules HeLa, on observe une bande majeure à 97 kDa ; pour les cellules HeLa en prolifération, des bandes supplémentaires de tailles inférieures à 97 kDa apparaissent (piste 2). Dans les fibroblastes pulmonaires humains à confluence, la protéine endogène n'est pas exprimée (piste 3) et
25 apparaît lorsque les cellules se mettent à proliférer (piste 4). Ces observations suggèrent que la protéine endogène ICBP90 est un marqueur de prolifération cellulaire pour des cellules normales (fibroblastes) tandis que pour les cellules tumorales elle serait un marqueur quelque soit le stade cellulaire.

30

Figure 2 : Immunoprécipitation de la protéine endogène

L'immunoprécipitation est réalisée sur des extraits protéiques totaux de cellules MOLT-4. L'anticorps 1RC1C-10 est fixé sur des billes de protéine G sépharose, puis mis en contact avec les extraits protéiques pendant 2 heures à température ambiante. Après lavage
5 les complexes billes/1RC1C-10/protéine sont précipités par centrifugation et analysés par migration sur gel de polyacrylamide 8% en présence de SDS, puis transferts sur membrane de nitrocellulose et révélation comme indiqué dans la figure 1. On
10 observe une bande unique de 97 kDa, ainsi qu'une bande de 45 kDa qui correspond à la chaîne lourde de 1RC1C-10.

Figure 3 : Localisation nucléaire de la protéine endogène

Nous avons utilisé des cellules HeLa pour examiner
15 l'expression endogène de la protéine ICBP90 in situ à l'aide de l'anticorps 1RC1C-10 et d'un anticorps secondaire anti-souris couplé au fluorochrome CY3. Le marquage est localisé exclusivement dans le noyau. Le nucléole et le cytoplasme ne sont pas marqués.

20

Figure 4 : Expression de l'ICBP59 endogène dans les cellules en prolifération

Nous avons observé la protéine endogène sur des coupes en paraffine d'appendice humain. Après déparaffinage et prétraitement
25 par chauffage en tampon acide (démasquage des sites antigéniques), les coupes sont incubées pendant 16 heures avec l'anticorps 1RC1C-10 dilué au 1/10000 (concentration initiale de 2 mg/ml). La révélation se fait par mise en contact avec un anticorps secondaire biotinylé, puis incubation avec le complexe
30 streptavidine-péroxydase. Une contre coloration des noyaux à

l'hématoxyline de Harris est également réalisée. Le marquage par 1RC1C-10 est localisé essentiellement dans des zones de prolifération cellulaire. Les cellules marquées se trouvent dans les cryptes glandulaires (CG) ainsi que dans les zones germinatives
5 (ger).

Figure 5 : Expression de ICBP-59 dans divers tissus humains

Nous avons évalué le niveau d'expression de l'ARNm correspondant à ICBP59 sur un dot blot d'ARN comportant 50
10 tissus humains différents. Le blot a été hybridé pendant 16 heures à 68° C avec une sonde d'ADNc radioactive (³²P) de 679 pb dans une solution d'hybridation ExpressHyb (Clontech). Après lavages, on réalise une révélation par autoradiographie (exposition une semaine à 80°C). Les tissus présentant le plus haut niveau
15 d'expression sont le thymus adulte et foetal, ainsi que la moelle osseuse adulte et le foie foetal.

Figure 6 : Séquence nucléotidique de ICBP90

L'ADNc codant pour ICBP90 comporte 2379 pb. Les portions
20 de séquence indiquées en gras sont celles qui n'apparaissent pas dans les bases de données d'EST humains (human dbest). Les autres parties de la séquence existent dans diverses EST:

de 1 à 325 : EST n° AI083773.
de 367 à 865 : EST n° AA811055.
25 de 940 à 1857 : EST n° AA488755, EST n° AA129794 et
EST n° AA354253.

Figure 7 : Séquence protéique de ICBP90

La séquence en acides aminés de ICBP90 est déduite par
30 traduction de la séquence nucléotidique de la figure 6. ICBP90

comporte 793 résidus et présente un poids moléculaire théorique de 89,758 kDa. Le p*K*_i est de 7,7. Les acides aminés indiqués en gras correspondent à ICBP-59.

5 Figure 8 : Détection de l'ICBP90 dans les sera de patients ayant des marqueurs sériques élevés de tumeurs solides.

Un volume de 2µl de sérum de chaque patient est dilué dans 1 ml de tampon PBS (Phosphate Buffer Saline 1X) contenant 0,1% de tween 20 suivi de dilutions croissantes réalisées dans le même tampon comme indiqué dans la figure. Un échantillon de 0,5 ml de chaque dilution est filtré sur la membrane de nitrocellulose à l'aide d'un appareil « Slot Blot BioRad ». La membrane est bloquée en présence de tampon PBS (contenant 0,1% de tween 20 et 5% de lait) pendant 1 heure à température ambiante. La protéine ICBP90 est révélée à l'aide de l'anticorps 1RC-1C10 (1ng/ml) et de l'anticorps secondaire (anti-souris couplé à la peroxydase dilué au 1/5000). Les bandes sont révélées par chimiluminescence par exposition pendant 10 secondes d'un film X-MAT (Kodak).

20 Figure 9 : Organisation structurale du gène ICBP90.

A . Des exons représentés par des boîtes : les boîtes grises représentent des exons codants ; des boîtes blanches représentent des exons non-codants. La taille des exons est indiquée en pb dans chaque boîte, et les noms des exons sont identiques au-dessus des boîtes. Les introns sont mentionnés de manière schématique par des lignes fines et leur taille approximative est indiquée en pb. Un site putatif de démarrage de la transcription et un signal consensus de polyadénylation sont indiqués. L'ATG est le codon de début de traduction et TGA le codon d'arrêt de la traduction.

B. Séquence de la région 5' flanquante du gène ICBP90 (Seq ID N° 12) (Numéro d'accèsion Genbank N° AF 220 226 déposée le 30 décembre 1999). Les exons sont en majuscules et les introns en minuscules. Le codon de début ATG est en majuscules gras, les
5 boîtes riches en GC (GC) et les boîtes CCAAT (CB) sont représentées en minuscules gras.

Figure 10 : Analyse du promoteur d'ICBP90

Les séquences du promoteur d'ICBP90 ont été fusionnées à
10 la séquence du gène « reporter » CAT dans le vecteur pBLCAT2 qui a ensuite été transfecté dans les cellules COS-1.

Une représentation schématique de ces constructions est représentée sur la gauche, avec le nombre se référant aux nucléotides en amont du codon d'initiation. Les activités CAT
15 relatives des extraits cellulaires correspondant à l'induction de l'activité du promoteur TK minimal sont exprimées en pourcentage (à partir de trois expériences de transfection indépendantes) et sont indiquées sur la droite.

20 **Figure 11 : Analyse par Northern Blotting et Western Blotting de l'expression d'ICBP90.**

A. L'hybridation Northern a été effectuée sur une membrane de Northern Blotting dont les dépôts d'ARN proviennent de lignées cellulaires cancéreuses de différents organes. Une sonde spécifique
25 d'ICBP90, synthétisée par PCR, et marquée à la digoxigénine, a été utilisée pour la détection des ARN_m d'ICBP90. Les tailles des ARN_m sont mentionnées sur la droite de la ligne 7.

Les lignes 1 à 7 contiennent des ARN provenant respectivement de la lignée leucémique promyélocytaire HL-60, de
30 Hela 53, de cellules K562 de leucémie myélogène chronique, de

cellules de leucémie lymphoplastique MOLT-4, de cellules Raji du lymphome de Burkitt, de cellules SW480 d'adénocarcinome colorectal, et de cellules A549 de carcinome pulmonaire.

L'histogramme montre les taux d'expression des ARN_m correspondant aux bandes de 5,1 kb et de 4,3 kb exprimés en pourcentage du taux d'expression de l'ARN_m de 5,1 kb des cellules HL-60 (ligne 1, figure 11A).

B. Analyse en Western Blotting de l'expression de ICBP90 dans les cellules MOLT-4 et Hela.

Des lysats de cellules totales de cellules Hela et MOLT-4 en prolifération ont été préparés. L'expression d'ICBP90 a été analysée en Western Blotting en utilisant l'anticorps 1RC1C-10.

EXEMPLE 1 : MISE EN EVIDENCE D'UNE NOUVELLE PROTEINE DE LIAISON A LA SEQUENCE ICB

1.1. Construction reportrice pour le criblage de la banque

Le système du simple hybride est une technique puissante qui permet de détecter *in vivo* chez la levure l'interaction de protéines avec des séquences d'ADN spécifiques en criblant des banques d'ADNc. Ceci permet d'évaluer directement l'ADNc correspondant de la protéine à lier. Plusieurs études ont permis d'identifier la nouvelle protéine dans cette méthode. Ces méthodes décrivent très bien les protocoles utilisés (Inouye *et al.*, 1994 ; Wang et Reed, 1993).

Brièvement, les oligonucléotides suivants ont été synthétisés 5'- AATTCGATTGGTTCTGATTGGTTCTGATTGGTTCIT-3' et 5'- CTAGAAGAACCAATCAGAACCAATCAGAACCAATCG-3'. Ces nucléotides sont ensuite hybridés. Selon les instructions du

fabricant (Clontech, Palo Alto, CA), la construction reportrice cible possède trois copies en tandem de la séquence ICB2 (ICB2X3). Comme mentionné plus haut, une copie de ICB2 est soulignée et les séquences CCAAT sont représentées en gras. Pour déterminer la

5 spécificité de liaison des protéines à la boîte ICB, les oligonucléotides suivants qui contiennent trois copies en tandem de la boîte GC1 (GC1X3) et également présents dans le promoteur ont été synthétisés et hybridés:

5'- AATTCGGGGCGGGGCCGGGGCGGGGCCGGGGCGGGGCT-3'

10 5'- CTAGAGCCCCGCCCGGCCCGGCCCGGCCCGGCCCGG-3'

Les fragments d'ADN cible résultant sont clonés dans le polylinker d'un plasmide intégratif pHISi-1 (Clontech) par ligation des extrémités cohésives au niveau du site EcoRI et XbaI, en amont du promoteur minimal du gène *his3*. La souche de levure YM4271

15 (Clontech) est utilisée pour la transformation et des colonies de levure ayant intégrées le plasmide dans leur génome sont sélectionnées sur un milieu synthétique Dropout ne contenant pas d'histidine. Deux clones ont été isolés : un pour ICB2 et un pour la boîte GC1.

20

1.2. Criblage de la banque

Une banque d'ADNc de la lignée cellulaire Jurkat clonée au site d'EcoRI du polylinker en aval de GAL4-AD du vecteur pGAD10 (Clontech) est utilisée pour le criblage selon les instructions du

25 fabricant. Des clones positifs sont sélectionnés puis cultivés sur un milieu sélectif dépleté en histidine et en leucine. L'ADN plasmidique de ces clones est récupéré et introduit par électroporation dans des bactéries *Escherichia coli* XL1-blue. Le séquençage des inserts a été réalisé sur une matrice d'ADN plasmidique purifiée à partir d'une

30 culture d'1,5 ml utilisant un kit de mini préparation (Bio-Rad,

Hercules, CA, USA). Une banque d'ADNc de thymus humain cloné dans λ gt10 (Clontech) a été criblée par hybridation sur une plaque, pour récupérer un ADNc codant la partie N-terminale de la protéine.

5

1.3. Découverte de ICBP-59

Les ADNc de quatre clones ayant rempli les critères de sélection du système simple hybride ont été séquencés, et les profils analysés à l'aide de bases de données informatiques (Genbank, 10 EMBL, PDB, Swissprot) afin de déterminer la nature des protéines codées. Deux clones correspondent à des protéines ribosomales (hRS12 et hRS4), un à une sérine-thréonine kinase (STPLK-1) et le quatrième à une protéine humaine d'un poids moléculaire théorique de 59 kDa (calculé à partir de la séquence traduite) et 15 non répertoriée.

Les ADNc, codant pour hRS4, hRS12 et ICBP-59 et obtenus par digestion par EcoRI des clones positifs obtenus dans le vecteur pGAD10, ont été clonés au site EcoRI du vecteur d'expression pGEX-4T-1 (Pharmacia). Les ADN recombinants sont ensuite 20 transformés dans une souche d'*Escherichia coli* adaptée (BL21). 500 ml de culture du clone sélectionné ont été utilisés lorsque une densité optique de 0,5 a été atteinte. La surexpression des protéines d'intérêt a été induite par l'IPTG (1mM) pendant 2h à 37° C. Le vecteur pGEX-4T-1 conduit à l'obtention de grandes quantités 25 de protéines sous forme fusionnée à la glutathion-S-transférase (GST). Les protéines de fusion avec la glutathione-S-transférase (GST) sont ensuite purifiées en utilisant des billes de sépharose couplé au glutathion (Pharmacia) suivi par une coupure durant la nuit avec de la thrombine (0,05 U/ml) à 4° C (Pharmacia).

Pour tester l'aptitude de la protéine de poids moléculaire de 59 kDa à lier spécifiquement les boîtes ICB1 et/ou ICB2, trois copies en tandem de ICB2 (ICB2X3, séquences décrites précédemment) ont été marquées au niveau terminal au phosphore ³²P en utilisant la polynucléotide kinase T4 (New England Biolabs) et du [γ ³²P]ATP (160 mCi/mmol, ICN Irvine, CA, USA). Pour examiner la spécificité de la liaison, des oligonucléotides contenant seulement une copie de la boîte CCAAT ont été synthétisés :

ICB1: 5'-AGTCAGGG**ATTGGCTGGTCTG**-3';

10 5'- CAGACCAG**CCAATCCCTGACT**-3'

ICB2: 5'-AAGCTACG**ATTGGTTCTTCTG**-3';

5'-CAGAAGA**CCAATCGTAGCTT**-3'.

La protéine ICBP-59 purifiée (1 μ g) est incubée avec 1 ng d'oligonucléotide marqué à son extrémité terminale par du phosphore ³²P dans 12% de glycérol, 12 mM d'HEPES-NaOH (pH 7,9), 60 mM KCl, 4 mM Tris-HCl (pH 7,9), 100 ng BSA, 0,6 mM DTT et 100 ng de poly(dI/dC) dans 20 μ l (Inouye et al., 1994). Après 30 minutes d'incubation à température ambiante, le mixte réactionnel est chargé sur des gels de polyacrylamide à 6%. Dans les expériences de compétition, la quantité indiquée d'oligonucléotides non marqués est ajoutée au mixte réactionnel 10 min avant l'addition de protéines. Pour examiner les propriétés de liaison de l'ICBP90 à la boîte ICB2, le même protocole est utilisé à la différence que l'oligonucléotide marqué contient seulement une

20

25 copie de la séquence CCAAT telle que décrite ci-dessous:

ICB2: 5'-ATAAAGGCAAGCTACG**ATTGGTTCTTCTGGACGGAGAC**-3'

5'-GTCTCCGTCCAGAAGA**CCAATCGTAGCTTGCCTTTTAT**-3'

La spécificité de liaison est étudiée en utilisant un nucléotide non marqué contenant une boîte GC du promoteur du gène de la topoisomérase II α humaine :

5'-GAATTCGAGGGTAAAGGGGGCGGGTTGAGGCAGATGCCA-3'

5 5'-TGGCATCTGCCTCAACCCCGCCCCCTTACCCTCGAATTC-3'.

Ces expériences de retard de migration sur gel d'acrylamide, nous ont permis de mettre en évidence que la nouvelle protéine humaine de 59 kDa est capable de lier une séquence d'ADN de type ICB, et ce de manière spécifique. Nous avons appelé cette protéine

10 ICBP-59 (pour Inverted CCAAT Box Binding Protein of 59 kDa).

EXEMPLE 2 : CARACTERISATION DE LA PROTEINE ICBP90

2.1. Synthèse d'anticorps

15 Les anticorps monoclonaux de souris sont synthétisés dans notre Laboratoire par injection de la protéine ICBP-59 par les méthodes traditionnelles (Brou *et al.*, 1993) ; la protéine a été au préalable purifiée par un système de fusion GST. Deux anticorps monoclonaux de 1RC1C-10 et 1RC1H-12 ont été sélectionnés pour

20 leur performance à détecter la protéine endogène correspondant à la protéine ICBP-59 à la fois dans des expériences de Western blotting et dans des expériences d'immunocytochimie. Avant utilisation, les anticorps sont purifiés sur colonne DEAE-cellulose (DE52, Whatmann) à partir des liquides d'ascites.

25

2.2. Mise en évidence de la protéine endogène par Western Blotting

Afin de détecter la protéine endogène correspondant à ICBP-59, nous avons dans un premier temps utilisé 1RC1C-10 en

30 Western blot (0,4 μ g/ml d'anticorps monoclonal 1RC1C-10) sur des

extraits nucléaires de cellules HeLa en situation de prolifération et de confluence (Figure 1). Les cellules COS-1 et HeLa sont cultivées tel que décrit précédemment (Brou *et al.*, 1993 ; Gaub *et al.*, 1998; Rochette-Egly *et al.*, 1997). Les cellules MOLT-4 sont cultivées dans
5 de l'air à 100% dans du RPMI supplémenté par 10% de sérum de veau fetal. Les fibroblastes pulmonaires humains en culture primaire sont préparés et cultivés dans du DMEM/F12 tel que décrit précédemment (Kassel *et al.*, 1998). Des extraits nucléaires de cellules Jurkat ont été achetés chez Sigma alors que ceux de
10 MOLT-4 et HL60 ont été préparés tel que décrit auparavant (Lavie *et al.*, 1999). Les cellules HeLa en phase de croissance et les fibroblastes pulmonaires humains sont obtenus par déplétion de la culture en sérum pendant 30 h suivi par la réintroduction pendant 16 heures par 10% de sérum de veau fétal (v/v). La prolifération est
15 arrêtée lorsque la confluence atteint 60 à 70%. Les cellules arrêtées à confluence (confluence de 100%) sont obtenues de manière concomitante en omettant l'étape de déplétion en sérum. Pour ces deux types cellulaires, des lysats cellulaires bruts sont préparés en récoltant les cellules dans du PBS (phosphate buffer saline) suivi
20 d'une étape de sonication. Pour les expériences d'immunotransfert des lysats de cellules totales et des extraits nucléaires sont chargés sur des gels de polyacrylamide SDS à 8% pour réaliser une électrophorèse en une dimension. Les protéines sont transférées sur des membranes de nitrocellulose bloquées avec un réactif de
25 blocage 10% (Roche Molecular Biochemicals, Mannheim, Germany) et incubées avec l'anticorps monoclonal purifié (1RC1C-10) à la concentration de 0,5 µg/ml. Un anticorps anti-souris de mouton couplé à la phosphatase alcaline (fragments Fab, Roche Molecular Biochemicals) est utilisé à une dilution de 1/2 500. Des signaux

sont détectés en utilisant le chlorure de 4-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate comme substrat.

Ces expériences montrent que la protéine endogène présente un poids moléculaire apparent d'environ 97 kDa. En outre, on observe que les formes de la protéine varie en fonction de la nature tumorale ou non-tumorale des cellules ainsi que de l'état de confluence ou de prolifération des cellules. En effet, dans les pistes correspondant aux extraits de cellules HeLa, on observe une bande majeure à 97 kDa ; pour les cellules heLa en prolifération, des bandes supplémentaires de tailles inférieures à 97 kDa apparaissent (piste 2). Dans les fibroblastes pulmonaires humains à confluence, la protéine endogène n'est pas exprimée (piste 3) et apparaît lorsque les cellules se mettent à proliférer (piste 4). Ces observations suggèrent que la protéine endogène ICBP90 est un marqueur de prolifération cellulaire pour des cellules normales (fibroblastes) tandis que pour les cellules tumorales elle serait un marqueur quelque soit le stade cellulaire.

L'utilisation de l'anticorps monoclonal dans des expériences d'immunoprécipitation sur des extraits de protéines nucléaires, suivies d'un Western blot, conduit de la même manière à la mise en évidence d'une protéine de 97 kDa (Figure 2).

Les résultats obtenus en Western blot, aussi bien pour les extraits de protéines nucléaires que pour les immunoprécipitations, montrent que la protéine de 59 kDa isolée à l'aide du système simple hybride ne constitue qu'un fragment de la protéine endogène humaine correspondante, en l'occurrence le fragment C-terminal à partir du résidu D263. Il nous a donc fallu entreprendre un nouveau criblage de banque d'ADNc.

2.3. Analyse en Dot Blot d'ARN de multiples tissus humains

Afin de choisir une banque nous donnant le plus de chance possible d'isoler la protéine complète, nous avons voulu identifier un tissu humain exprimant l'ARN messager (ARNm) correspondant en quantité importante. A l'aide d'une sonde d'ADNc recouvrant une partie de la séquence de ICBP59 et marquée au ^{32}P , nous avons testé l'expression de l'ARNm d'intérêt dans 50 tissus humains différents sur un dot blot d'ARN. Brièvement, une sonde longue de 678 paires de bases correspondant à la séquence en acides aminés 269 à 500 de ICBP90 a été synthétisée par PCR en utilisant de la Taq polymérase (Sigma, St Louis, MO, USA). La sonde marquée par random priming en utilisant du dCTP- α ^{32}P est purifiée sur colonnes Sephadex G50 (Pharmacia, Uppsala, Suède).

Un dot blot d'ARN de multiples organes contenant de l'ARN poly(A)⁺ de 50 tissus humains différents est hybridé 20 heures dans des conditions de forte stringence dans un milieu ExpressHyb (Clontech) à 68° C avec une sonde marquée au ^{32}P . Des lavages à haute stringence sont réalisés dans du 0,1 x SSC, 0,1% SDS à 68° C (De Vries *et al.*, 1996).

Les résultats obtenus (figure 5) montrent que les tissus exprimant le plus fortement l'ARNm de la protéine ICBP-59 sont le thymus adulte et fœtal, ainsi que la moelle osseuse adulte et le foie fœtal. Pour isoler la protéine entière, notre choix s'est donc porté sur une banque d'ADNc de thymus adulte.

2.4. Criblage de la banque et clonage de ICBP90

Le criblage de la banque nous a permis d'obtenir plusieurs clones d'environ 4000 paires de bases (pb) comportant un cadre de lecture ouvert de 2379 pb (Fig.6). Cette séquence code pour une protéine de 793 acides aminés (Fig.7) dont le poids moléculaire

théorique (calculé à partir de la séquence traduite) est de 89,758 kDa. Nous avons appelé cette protéine ICBP90 (pour Inverted CCAAT Box Binding Protein of 90 kDa) par analogie avec l'appellation utilisée pour la protéine initiale de 59 kDa.

- 5 L'ADNc ICBP90 (2379 bp) a été synthétisé par PCR en utilisant l'ADN polymérase Deep Vent (New England Biolabs, Beverly, MA, USA) et les oligonucléotides utilisés au cours de cette réaction de PCR étaient voisins du site de EcoRI. Le produit de réaction a été par la suite souscloné dans un vecteur pGEX-4T-1
10 (Pharmacia) pour l'expression de la protéine de fusion GST dans BL21. La surexpression est induite par IPTG (1mM) pendant 4h à 25° C. La protéine ICBP90 est ensuite purifiée.

2.5. Immunocytochimie et immunohistochimie.

- 15 L'observation directe de la protéine ICBP90 sur des cellules et tissus a été également mise en œuvre.

- Des cellules COS-1 ont été transfectées comme décrit précédemment (Brou *et al.*, 1993 ; Gaub *et al.*, 1998) avec le vecteur pSG5 (Stratagene, La Jolla, CA) dans lequel l'ADNc de ICBP90
20 (2379 bp) a été sous-cloné dans le site de restriction EcoRI. L'ADNc est synthétisé par réaction de polymérisation en chaîne (PCR) utilisant la polymérase Deep Vent (New England Biolabs) et des oligonucléotides flanquants le site de restriction EcoRI. La construction plasmidique est vérifiée par séquençage.
- 25 L'immunomarquage des cellules HeLa et des cellules COS-1 transfectées est réalisé tel que décrit précédemment (Brou *et al.*, 1993) avec respectivement les anticorps monoclonaux 1RC1C-10 et 1RC1H-12. Un marquage indirect à l'immunopéroxydase de ICBP90 et de topoisomérase II α est réalisé comme décrit précédemment (Rio
30 *et al.*, 1987, Devys *et al.*, 1993). Les appendices humains ont été

inclus dans la paraffine et fixés dans du formalin 10% tamponé (Sigma). Des coupes sériées (3 µm) sont incubées durant la nuit à température ambiante avec l'anticorps IRC1C-10 et avec l'anticorps anti-topoisomérase IIα (NeoMarkers, Union City, CA, USA). Des anticorps liés de manière spécifique sont visualisés par un complexe utilisant la streptavidine biotine (LAB/LSAB method, 5 Dako LSAB2 System kit ; DAKO, Carpinteria, CA, USA).

En immunocytochimie, l'anticorps IRC1C-10 marque le noyau des cellules HeLa tandis que le nucléole et l'ensemble du cytoplasme ne sont pas marqués (Figure 3). En 10 immunohistochimie, des coupes en paraffine d'appendice humain montrent un marquage localisé essentiellement dans des zones de prolifération cellulaire (Figure 4). En effet, les cellules marquées sont logées dans les cryptes glandulaires (CG) ainsi que dans les zones germinatives (Ger). Un marquage identique est obtenu 15 lorsqu'on utilise un anticorps anti-topoisomérase IIα qui est une enzyme uniquement exprimée dans des cellules en prolifération (résultats non illustrés).

20 2.6. Recherches BLAST et prédiction de domaines

Les études sur BLAST en ligne ont été réalisées à partir des informations du National Center for Biotechnology Information au National Institute of Health (Bethesda, MD, USA). SCANPROSITE et PROFILESCAN sont utilisés pour l'analyse protéique (Infobiogen, 25 Villejuif, France).

ICBP90 comporte un domaine « ubiquitin-like » dans ses 80 premiers acides aminés, deux sites de localisation nucléaires potentiels dans la partie C-terminale et deux domaines en doigt de zinc (« zinc-finger »), dont l'un serait impliqué dans la liaison à 30 l'ADN et l'autre dans des interactions protéine-protéine. Plusieurs

sites potentiels de phosphorylation par la protéine kinase C, la caséine kinase II ainsi que par une tyrosine kinase sont également présents.

La production et la purification de ICBP90 à l'aide du système
5 de fusion GST (même procédé que celui utilisé pour ICBP-59) nous a finalement permis de tester la capacité de la protéine complète à lier les séquences d'ADN de type ICB. Son comportement est en tous points identique à celui observé pour ICBP-59.

En définitive, nous avons isolé une nouvelle protéine
10 humaine que nous avons appelée ICBP90 pour les raisons évoquées ci-dessus. Son poids moléculaire théorique est de 89,758 kDa et son poids moléculaire apparent sur gel d'acrylamide est de 97 kDa. Cette protéine est non seulement localisée exclusivement dans le noyau des cellules humaines, mais elle présente également la
15 capacité à lier des séquences d'ADN de manière spécifique, en l'occurrence des séquences de type CCAAT. Pour ces raisons, nous pensons que ICBP90 a la possibilité de moduler l'expression des gènes dont le promoteur est pourvu de boîtes CCAAT, éventuellement en position inversée (ICB). Le gène de la
20 topoisomérase II α humaine qui nous intéresse plus particulièrement, et qui comporte cinq séquences ICB dans son promoteur, nous semble être une des cibles privilégiées de ICBP90.

Ces expériences ont permis de mettre en évidence les caractéristiques remarquables de l'anticorps 1RC1C-10 qui ne
25 marque uniquement que les cellules en prolifération dans le cas de cellules non cancéreuses ; il marque les cellules cancéreuses en prolifération ou en quiescence ; il est utilisable dans 4 techniques différentes (Western blotting, immunocytochimie, immunohistologie, immunoprécipitation) ; il possède une très
30 bonne affinité et permet d'utiliser une dilution de 1/150 000 en

immunohistochimie (i.e. 13 ng/ml) ; enfin, son utilisation ne génère quasiment pas de bruit de fond.

Les applications futures de IRCIC-10 se situent en premier lieu dans les domaines du diagnostique et de la recherche fondamentale. Pour le diagnostique en anatomo-pathologie par exemple, il serait tout à fait possible de rendre compte de l'état prolifératif d'un tissu cancéreux donné. Concernant la recherche fondamentale, des investigations sont en cours dans notre laboratoire afin de déterminer la contribution exacte de ICBP90 dans les mécanismes de prolifération des cellules normales et des cellules cancéreuses. Or, pour l'étude de l'expression de ICBP90 en fonction du cycle cellulaire, de sa localisation nucléaire précise et de son interaction avec d'autres protéines cellulaires, l'utilisation de l'anticorps sera incontournable.

Pour l'instant nous n'avons pas étudié l'expression de l'ICBP90 en fonction du cycle cellulaire. Néanmoins, dans le cas où des lignées de cellules cancéreuses sont confluentes ou lorsqu'elles sont en prolifération nous ne pouvons détecter de différences significatives de l'expression de l'ICBP90 (Fig. 1) du moins en ce qui concerne la forme à 97 kDa. Par contre, dans des cellules confluentes non cancéreuses (cellules musculaires lisses bronchiques humaines) l'expression de l'ICBP90 est difficilement détectable (résultats non illustrés). Ceci est confirmé sur les coupes histologiques où aucune cellule en quiescence n'est marquée par l'anticorps. Il est par conséquent possible que l'ICBP90 soit exprimée quelle que soit la phase du cycle cellulaire dans des cellules cancéreuses alors que son expression varierait en fonction de chaque phase dans des cellules non cancéreuses. Ceci rend donc l'utilisation de l'anticorps extrêmement intéressante, en ce sens que nous aurions à disposition un marqueur de la

prolifération cellulaire de tissus cancéreux qui ne dépendrait pas de la phase du cycle cellulaire contrairement à d'autres marqueurs de prolifération cellulaire tels que le Ki-67, la topoisomérase II α , la cycline E et la cycline B1. En effet, la fin de la phase S est caractérisée par une très faible expression de Ki-67, la cycline E
5 marque les cellules en fin de phase G1 jusqu'au milieu de la phase S et la cycline B1 marque les cellules en phase G2/M (pour revue Darzynkiewicz *et al.*, 1994). Par ailleurs, il a été montré que PCNA (Proliferating Cell Nuclear Antigen) surestime le nombre de cellules
10 en prolifération dans certains types de tissus (Roskell and Biddolph, 1999).

ICBP90 joue un rôle important dans la prolifération cellulaire en régulant l'expression de gènes tels que celui de la topoisomérase II α . Différentes stratégies visant à bloquer l'action de cette protéine
15 doivent permettre de modifier la prolifération cellulaire. Ainsi, l'utilisation de l'anticorps IRCIC-10 ainsi que l'utilisation de peptides mimant l'interaction ADN/ICBP90 sans pour autant engendrer d'effet physiologique subséquent constitue une possibilité intéressante. Le design de ses peptides s'inspirerait
20 directement de la séquence protéique de ICBP90 que nous avons décrite. Une forme tronquée correspondant à ICBP59 pourrait par exemple être un des premiers candidats.

Le blocage pur et simple de l'expression de ICBP90 dans le but d'éliminer complètement son influence sur les gènes et par
25 extension sur la prolifération cellulaire peut être envisagé ; il peut se faire soit par une approche classique en obtenant des inhibiteurs de la protéine, soit en utilisant une approche plus moderne correspondant à la technique d'interférence par l'ARN double brin (RNA interférence ou RNAi) telle que décrit récemment par
30 Kennerdell & Carthew (1998).

EXEMPLE 3 : ISOLEMENT ET CARACTERISATION DU GENE ICBP90

5 3.1. Matériels et méthodes

3.1.1. Construction et criblage d'une banque génomique placentaire humaine

Après digestion partielle avec l'enzyme MboI, l'ADN
10 génomique placentaire a été fractionné en fonction de la taille sur
un gradient de 10 à 40% de sucrose. Des fragments d'ADN de
15 kb ont été ligués dans un vecteur λ GEM12 préalablement digéré
par BamHI (Promega, Madison WI, USA). Après empaquetage, les
particules de phages λ ont été titrées sur des cellules TAP 90. La
15 banque génomique contient 3.10^6 unités formant des plaques
(plaques forming units, pfu). 10^6 clones ont été étalés pour
l'analyse. Une sonde de 620 pb correspondant à une extrémité 5'
terminale de l'ADN_c de ICBP90 utilisée pour le criblage a été
marquée au $\alpha^{32}\text{P}$ -dCTP par une méthode d'amorçage aléatoire
20 (random priming) (Sambrook *et al.*, 1989). La sonde marquée est
utilisée selon un protocole classique d'hybridation sur plaque pour
cribler la banque génomique (Sambrook *et al.*, 1989). L'hybridation
a été réalisée à 68°C dans du 5X SSC (15 mM NaCl, 1,5 mM citrate
de sodium pH 7,0), 5 X de solution Denhardt, 100 μg / ml d'ADN de
25 sperme de saumon et 0,1% de SDS, suivi par 30 minutes de
lavages dans du 2X SSC, 0,1% SDS à température ambiante.

Deux étapes de criblage ont été réalisées pour purifier un
clone positif. Le clone positif a ensuite été digéré avec l'enzyme NotI
et deux fragments de 6 et de 10 kb ont été sous-clonés dans le

vecteur pBluescript-SK⁺ (Stratagène, La Jolla CA, USA) selon un protocole standard (Sambrook *et al.*, 1989).

3.1.2. Criblage de la banque d'ADN_c de thymus humain

5

Une banque λ GT10 d'extrémité 5' d'ADN_c de thymus humain (Clontech, Palo Alto, CA, USA) a été criblée par hybridation sur plaque en utilisant la sonde d'ADN_c de 679 pb synthétisée telle que dans le paragraphe relatif à l'analyse par Northern Blotting. Des
10 signaux ont été détectés en utilisant du chlorure de 4-nitro-bleu-tétrazolium et de 5-bromo-4-chloro-3-indolyl-phosphate comme substrat.

3.1.3. Réaction de polymérisation en chaîne (PCR) sur 15 l'ADN génomique placentaire

L'ADN génomique placentaire a été préparé selon une méthode conventionnelle (Sambrook *et al.*, 1989). Pour la région 5' du gène ICBP90, les inventeurs ont utilisé le kit PCR Advantage[®]-
20 GC genomic de Clontech qui est adapté aux régions riches en GC de l'ADN génomique. Pour couvrir les régions 3'-flanquantes, la Taq polymérase (Sigma, St Louis, MO, USA) et son tampon correspondant ont été utilisés. Les réactions ont été réalisées selon les instructions du fabricant en utilisant 250 ng d'ADN génomique
25 placentaire comme matrice dans un volume final de 50 μ l. Afin d'obtenir l'amplification des introns de longueur 19 kb et 8,7 kb le système PCR Expand[™] 20kb^{plus} (Roche Diagnostics, Mannheim, Germany) a été utilisé.

La réaction a été réalisée dans 100µl en utilisant 125 ng d'ADN génomique placentaire par réaction.

3.1.4. Constructions plasmidiques et essais CAT

5 Une série de différents fragments ont été obtenus par PCR dans la région 5' flanquante du gène ICBP90 en utilisant des amorces de 20 nucléotides afin d'obtenir les constructions décrites dans la figure 10. Celles-ci contiennent un site de restriction
10 BamHI et l'ADN génomique placentaire humain a été utilisé comme amorce. Les produits PCR ont été digérés et sous-classés en amont du gène reporter chloramphénicol acétyl transférase (CAT) d'un vecteur contenant le promoteur minimal de la thymidine kinase (pBICAT2). Les constructions plasmidiques ont été vérifiées par
15 séquençage. Des cellules COS-1 ont été cultivées dans un milieu Dulbecco modifié par Eagle (DMEM) supplémenté avec 5% de sérum de veau fœtal. Après l'étalement, les cellules ont été transférées avec les différentes constructions plasmidiques (5 µg) en utilisant la technique de co-précipitation au phosphate de calcium
20 (Banerji *et al.*, 1981)). Les analyses d'expression de la CAT ont ensuite été réalisées comme décrit ailleurs (Goetz *et al.* (1996)).

3.1.5. Localisation chromosomique du gène ICBP90

25 Des chromosomes métaphysiques ont été préparés à partir de leucocytes humains du sang périphérique selon les protocoles standards (Haddad *et al.* (1988)). Brièvement, une sonde de 10 kb correspondant à un fragment 5' terminal du clone de 16 kb isolé à partir du criblage de la banque d'ADN génomique placentaire, a été
30 marquée avec de la biotine-16-dUTP (Roche Diagnostics) par « nick-

translation ». La sonde est ensuite précipitée avec un excès (50X) d'ADN humain Cot-1 (Life Technologies, Rockville MD), resuspendu dans 50% de formamide, 1X SSC, pré-hybridé pendant 2 heures à 37°C puis hybridé sur la nuit à 37°C. La détection est réalisée en utilisant de l'avidine-FITC (Vector Laboratories, Burlingame CA). Les chromosomes ont été contre-colorés avec du 4'-6-diamino-2-phénylindole (Sigma).

3.1.6. Analyse de Northern blotting et de Western Blotting

Une membrane de Northern Blotting contenant 2 µg d'ARN polyA⁺ par ligne, provenant de 7 lignées cellulaires humaines cancéreuses différentes (Clontech) a été préhybridée dans du Express Hyb (Clontech) puis hybridée avec la sonde spécifique de ICBP90 dans du Express Hyb à 68°C pendant deux heures. La sonde double-brin marquée à la digoxigénine a été préparée par amplification PCR d'un fragment de 676 pb à partir de l'ADNc d'ICBP90 (nucléotides 806 à 1 485 ; Numéro d'Accession Genbank AF 129 507) selon les instructions du fabricant (Roche Diagnostics).

Après purification au travers d'une colonne de chromatographie Micro Bio-Spin 30 (Bio-Rad, Hercules, CA), la sonde spécifique ICBP90 (5 ng/ml) a été chauffée à 95°C pendant 15 minutes puis refroidie dans la glace avant l'addition de la solution d'hybridation. Les lavages après l'hybridation ont été réalisés deux fois dans du 2X SSC, 0,1% SDS (30 minutes par lavage à température ambiante), puis deux fois dans du 0,1X SSC, 0,1% SDS (30 min. par lavage à 68°C). La membrane a été traitée avec la solution A (0,1 M acide malique, 0,15 M de NaCl à pH 7,5)

puis bloquée par incubation avec 1% d'agent bloquant (Roche Diagnostics) dans le tampon A pendant 30 minutes à température ambiante.

Un anticorps conjugué à la phosphatase-alcaline dirigé
5 contre la digoxigénine (fragment Fab, Roche Diagnostics) a été
ajouté (150 mU/ml) puis incubé pendant 30 minutes à température
ambiante. La membrane a ensuite été lavée deux fois avec la
solution A puis équilibrée dans du 0,1 M tris-HCl, 0,1 M NaCl, pH
9,5. Pour la détection par chémiluminescence, les inventeurs ont
10 utilisé l'agent Disodium 3-(4-méthoxyspiro{1,2 dioetane-3,2'-(5'-
chloro) tricyclo [3.3-1.1^{3,7}] décan }-4-yl) phényl phosphate® (Roche
Diagnostics) selon les instructions du fabricant. Les bandes d'ARN_m
ont été quantifiées en utilisant le logiciel NIH image 1.62 et
exprimées en pourcentage de la bande d'ARN_m la plus abondante
15 (c'est-à-dire la bande de 5,1 kb des cellules HL-60).

L'analyse en Western Blotting a été réalisée comme décrit
ailleurs (Hopfner *et al.* (2000)). Les signaux ont été détectés en
utilisant le chlorure de 4-nitro-blue tétrazolium / le phosphate de
5-bromo-4chloro-3-indolyl comme substrat.

20

3.1.7. Outils de recherche d'alignement local de base, prédictions de sites de démarrage de la transcription et de signal polyA

25 Des recherches d'alignement local de base ont été réalisées
via le Centre National d'Information en Biotechnologie au National
Institute of Health (Bethesda, MD, USA). Le criblage d'une banque
de facteurs de transcription avec le programme d'ordinateur Mat
Inspector, les prédictions de sites de démarrage de la transcription
30 (TSS) avec Neural Network, ainsi que la prédiction de signal polyA,

ont été réalisés via le Baylor College of Medicine (Reese *et al.* (1996)).

3.2. Résultat

5

3.2.1. Isolement et caractérisation du gène de l'ICBP90

Une banque d'ADN complémentaire de placenta humain cloné dans le phage lambda GEM 12 a été criblée à l'aide d'une
10 sonde d'ADN. Le criblage a conduit à la purification d'un seul clone positif ayant un insert de 16 kb. L'analyse de la séquence a permis de déterminer qu'il contenait une séquence intronique longue de 10 kb et contenant 3 exons (appelé B, C et D dans la figure 9A). Tous les autres criblages, incluant notamment ceux qui ont été réalisés
15 par PCR sur des banques de BAC (Bacterial Artificial Chromosome) ou de YAC (Yeast Artificial Chromosome), n'ont pas permis d'isoler d'autres clones positifs. Par conséquent, nous avons décidé de déterminer le reste de l'organisation du gène par PCR directement sur de l'ADN génomique de placenta humain. La plus grande
20 difficulté fut d'obtenir le côté 5' de l'intron de 19 kb. Ainsi, des amorces ont été choisies dans l'exon A (amorce sens) et dans le côté 5' du clone de 16 kb (amorce anti-sens). L'exon E et l'intron de 8,7 kb ont été amplifiés en utilisant une amorce sens dans l'exon D et l'amorce anti-sens dans l'exon F. Finalement, la séquence complète
25 de l'exon F jusqu'au signal de poly-adénylation a été déterminée en utilisant une amorce sens choisie dans le début de l'exon F et l'amorce anti-sens dans le côté 3' d'une EST (référence dans GenBank n° AW297533) homologue à la séquence du gène de l'ICBP90. La séquence complète du gène de l'ICBP90 montre qu'il
30 est composé de 6 exons codants dont la taille varie de 100 paires de

bases à 3453 paires de bases. La plupart des jonctions exons/introns répondent aux séquences consensus pour les sites accepteurs et donneurs d'épissage. Une séquence consensus de poly-adénylation (AATAAA) a été trouvée dans la région 3', c'est-à-dire 1152 nucléotides après le codon stop dans la figure 9A.

3.2.2 La région 5' du gène de l'ICBP90

Le criblage d'une banque d'ADN complémentaire de thymus humain cloné dans le phage lambda gt10 a conduit à l'obtention de deux populations de cDNA qui se distinguent l'une de l'autre dans leur région 5', exactement 10 paires de bases en amont du codon d'initiation, c'est-à-dire dans la région 5' non traduite. Ces deux populations de cDNA prédisent l'existence de deux exons alternatifs en 5' appelés exon I et exon II (Figure 9A). Nous avons observé que les exons I et II sont reliés à un site d'épissage alternatif interne de l'exon A. De plus, nous avons trouvé dans une base de données un EST (référence dans GenBank n° AI084125) correspondant aux nucléotides 1290 à 1356 (Figure 9B). Les positions de ces deux exons et de l'EST à l'intérieur du locus ont été déterminées par PCR. Pour cela nous avons utilisé des amorces correspondant aux 18 premiers nucléotides de chaque exon et une amorce anti-sens choisie dans le premier exon traduit (exon A). Cette stratégie nous a permis de reconstruire la région 5' telle qu'elle est représentée dans les figures 9A et 9B, avec l'exon I correspondant aux nucléotides 1 à 134 et l'exon II correspondant aux nucléotides 676 à 725. La séquence EST (AI084125) est contiguë au site d'épissage interne de l'exon A. Nous n'avons pas encore déterminé avec précision le début des exons I, II et A puisque leurs séquences ont été déduites à partir de criblages de banques de cDNA (Figure 9A).

Quatre boîtes GC (GC1 à GC4) ont été trouvées dans la région 5' (Figure 9B). Ces boîtes représentent des sites potentiels de liaison pour le facteur de transcription Sp1, mais seulement une boîte (GC3) correspond à une séquence consensus, c'est-à-dire GGGGCGGGG. De plus deux boîtes CCAAT (CB1 et CB2) ont été trouvées. Des analyses prédictives de séquences suggèrent que deux régions promotrices existent dans la région 5', c'est-à-dire avant le codon d'initiation (ATG). Deux sites potentiels d'initiation de la transcription ont été prédits aux positions 571 et 827. Le premier suit la séquence consensus de liaison à Sp1 et le second suit la boîte GC1 (respectivement entre les exons I & II, et les exons II & A). Afin de voir si ces deux régions sont fonctionnelles en tant que région promotrice, plusieurs constructions plasmidiques contenant un gène rapporteur (gène de la Chloramphénicol Acétyl Transférase; CAT) en aval des différentes régions promotrices potentielles ont été préparées. Des cellules COS ont été transfectées avec ces constructions plasmidiques. La figure 10 montre les résultats obtenus et qui correspondent au pourcentage d'augmentation de l'activité basale. L'activité maximale a été obtenue avec la construction plasmidique contenant 1114 paires de bases en amont du site d'initiation de la traduction, avec une augmentation de 236,7% de l'activité promotrice basale (promoteur minimal du gène de la thymidine kinase). La construction plasmidique contenant 642 paires de bases en amont de l'ATG a conduit à une augmentation de 115,6% alors que la construction plasmidique contenant uniquement la séquence entre l'exon I et l'exon II montrait une activité relativement faible avec une augmentation uniquement de 22,8% (figure 10). Ces résultats suggèrent l'existence d'une région promotrice entre les exons II et A.

3.2.3. Localisation chromosomique du gène ICBP90

La localisation chromosomique du gène ICBP90 a été réalisée par hybridation *in situ* de fluorescence (FISH). Le gène ICBP90 est
5 localisé sur le chromosome 19p13.3 dans une région télomérique. Une recherche réalisée dans Genbank a montré qu'une région de 6Mb dans la bande chromosomique 19 p 13.3 d'une banque de cosmide spécifique du chromosome 19 (hybride homme / hamster 5HL2-B) contient 147 nucléotides codant pour les acides aminés
10 746 à 793 de ICBP90. Cette séquence a été localisée entre les marqueurs STS (sequence tagged site) D19S883 et D 19S325.

3.2.4 Expression de ICBP90 dans différentes lignées cellulaires

15

ICBP90 participe à la régulation de l'expression du gène TopII α (Hopfner *et al.* (2000)). Comme TopII α est exprimée de manière différentielle dans différentes tumeurs et lignées cellulaires, ICBP90 lui-même est susceptible d'avoir une régulation
20 complexe en terme d'activité et d'expression génique.

Dans une première étape vers la compréhension des mécanismes régulant l'expression du gène ICBP90, l'ARN_m d'ICBP90 a été analysé dans différentes lignées cellulaires. L'ARN_m d'ICBP90 a été étudié dans la lignée cellulaire HL60 dérivée d'une
25 leucémie promyélocytaire (ligne 1), de cellule Hela S3 (ligne 2), de cellules de leucémie lymphoblastique MOLT-4, de cellules Raji du lymphome de Burkitt (ligne 5), d'adénocarcinome colorectal SW 480 (ligne 6), de cellules A549 de carcinome du poumon (ligne 7) (figure 11A).

Deux bandes d'ARN_m de 4,3 et 5,1 kb sont observées. Les quantités relatives d'ARN_m dans les bandes varient selon le type cellulaire. L'histogramme de la figure 11A montre les taux d'ARN_m dans les bandes de chacune des lignées cellulaires, exprimé en pourcentage de la quantité maximale observée de bandes d'ARN_m de 5,1 kb dans les cellules HL-60 (ligne 1, figure 11A). Dans les cellules MOLT-4, seule la bande d'ARN_m de 4,3 kb est observée, alors que dans les cellules de leucémies promyélocytaires la bande à 5,1 kb est prédominante. Dans les cellules Raji du lymphome de Burkitt, seule la bande à 5,1 kb est détectée. Approximativement, des quantités égales des deux types d'ARN_m sont observées dans les autres lignées cellulaires, c'est-à-dire les cellules Hela, K562, A549, SW 480. Pour les cellules HL-60, néanmoins, l'ARN_m de 5,1 kb est plus fortement exprimé que l'ARN_m de 4,3 kb. D'autres analyses ont été entreprises sur les cellules Hela pour confirmer que les 2 transcrits proviennent de la transcription du gène ICBP90. Une sonde d'ADN_c de 626 pb marquée à la digoxigénine localisée immédiatement en amont du signal poly A (c'est-à-dire l'exon F) et utilisée comme sonde pour des expériences de Northern Blotting, a produit les mêmes résultats, c'est-à-dire l'apparition de deux bandes d'ARN_m de 4,3 kb et de 5,1 kb. Ce résultat confirme que les deux formes d'ARN_m sont générées à partir d'un seul gène.

Les inventeurs ont également étudié l'expression de la protéine ICBP90 afin de déterminer si ces deux isoformes d'ARN_m sont susceptibles de coder pour deux protéines différentes.

La figure 11B montre le profil d'expression de ICBP90 dans des extraits protéiques de cellules MOLT-4 et Hela. Alors qu'une seule bande de 97 kDa est observée dans les cellules MOLT-4, dans les cellules Hela, à côté de la bande de 97 kDa qui est doublée, plusieurs autres bandes d'un poids moléculaire inférieur sont

observées. Ces résultats suggèrent que, dans les cellules MOLT-4, un ARN_m code pour une forme unique d'ICBP90. A l'inverse, dans les cellules Hela, les deux ARN_m sont susceptibles de conduire à la production de différents isoformes d'ICBP90.

5

3.3 Commentaires

Le gène ICBP90 s'étend sur environ 35,8 kb. Six exons traduits et deux exons non traduits, et de fait sept introns, ont été
10 identifiés par les inventeurs. Les deux domaines en doigt de zinc de ICBP90 sont codés par le même exon (exon F), contrairement au gène du récepteur aux œstrogènes humains dans lequel chacun des doigts-de-zinc putatifs du domaine de liaison à l'ADN du récepteur sont codés séparément (Ponglikitmongkol *et al.* (1988)).
15 Le domaine « ubiquitin-like » de ICBP90 est codé par les exons A et B alors que le domaine « leucine zipper » est codé par l'exon B. De manière intéressante, l'exon F seul est susceptible de coder pour une protéine fonctionnelle car elle code pour deux signaux de localisation nucléaire, les domaines zinc-finger et plusieurs sites
20 putatifs de phosphorylation. Deux grands introns de 8,7 kb et de 19 kb ont été trouvés.

Le gène ICPB 90 a été localisé dans la région chromosomique 19p13.3. Plusieurs autres gènes ont été localisés dans cette région, par exemple le Nuclear Factor I/C (également un facteur de
25 transcription liant CCAAT, (Qian *et al.* (1995)). De manière intéressante, une translocation atypique t (7 ; 19) dans la leucémie myélomonocytaire aiguë, impliquant un site fragile au locus 19p13.3 a été décrite (Sherer *et al.* (1991)). Egalement, il a été suggéré que les gènes impliqués dans le développement des
30 carcinomes pancréatiques sont localisés en 19p13.3 et 19q13.1-

13.2 (Hoglund *et al.* (1998)). Des réarrangements des bandes 14q32.3 et 19p13.3 d'une délétion préférentielle du bras court du chromosome 1 constituent des altérations chromosomiques non aléatoires dans le myélome multiple et la leucémie des cellules du plasma (Taniwaki *et al.* (1996)). D'autres gènes ont été localisés dans cette région ; ils incluent un gène impliqué dans l'adénocarcinome du syndrome Peutz-Jeghers (Gruba *et al.* (1998)). Egalement, il a été suggéré que le gène suppresseur de tumeur putatif pour l'adénome malin est localisé en D19S216 au niveau de la bande chromosomique 19p13.3 qui joue un rôle important dans la tumorigenèse de l'adénome malin (Lee *et al.* (1998)).

L'analyse de la séquence de la région 5' du gène ICBP90 a révélé l'existence de plusieurs exons non-traduits avec une région promotrice entre les exons II et A et probablement un second promoteur plus faible localisé entre les exons I et II. La région promotrice entre les exons II et A est un promoteur sans séquence TATA, suggérant que le gène ICBP90 peut être un gène de ménage, au moins lorsque ce promoteur est impliqué. En ce sens, il ressemble fortement aux régions promotrices des gènes ATF α (Goetz *et al.*, 1996), CRE-BP1 / ATF 2 (Nagase *et al.*, 1990) et TopII α , (Hochhauser *et al.*, 1992), qui ne contiennent pas de boîtes TATA canoniques mais plusieurs sites de liaison de SP-1.

Les boîtes GC et/ou CCAAT sont susceptibles d'être impliquées dans la régulation de l'expression du gène ICBP90 via les facteurs de transcription SP-1 et les protéines de liaison à CCAAT. De plus, étant donné que la protéine ICBP90 est une protéine de liaison à CCAAT, ICBP90 est également susceptible de réguler sa propre expression.

Une banque de données de facteurs de transcription a été criblée à l'aide du programme d'ordinateur Mat Inspector du Baylor

College of Medicine, et de nombreux sites de liaison de facteurs de transcription ont été identifiés dans la séquence précédant le codon ATG (figure 9B). Parmi ces sites de liaison aux facteurs de transcription, il est intéressant de noter des sites de liaison du

5 facteur de transcription AP-2 régulé au cours du développement et qui contrôle l'expression de gène tel DR-nm 23 (Martinez *et al.* (1997)), les sites de liaison de la protéine myéloïde « zinc-finger » MZF 1 qui est impliquée dans la régulation de l'hématopoïèse (Hromas *et al.* (1996)).

10 L'analyse de Northern Blotting a démontré qu'il existe deux populations d'ARN_m de 4,3 kb et de 5,1 kb. De manière intéressante, chaque population présente une spécificité cellulaire. Par exemple, les cellules lymphoblastiques MOLT-4 expriment seulement l'ARN_m de 4,3 kb, alors que dans les cellules Raji du

15 lymphome de Burkitt (lymphocytes B matures), seul le transcrit de 5,1 kb est observé. Les cellules HL-60 expriment davantage d'ARN_m de 5,1 kb que d'ARN_m de 4,3 kb. Les cellules HL-60 et les cellules Raji du lymphome de Burkitt sont davantage différenciées que les cellules MOLT-4 suggérant que les taux d'expression du transcrit

20 de 5,1 kb par rapport à celui de 4,3 kb peut être directement corrélé avec l'état de différenciation des cellules.

De manière intéressante, une étiquette de séquence exprimée (EST, Expressed Sequence Tag) correspondant à la séquence 5' de l'exon A a été identifiée à partir d'oligodendrogliome anaplastique

25 (numéro d'accèsion Genbank N° AI 084 125) alors qu'une EST correspondant à l'inclusion de l'exon II a été isolée à partir d'un mélange de tumeurs de cellules germinales (numéro d'accèsion Genbank N° AI 968 662). Les résultats des inventeurs suggèrent donc que la régulation des transcrits ICBP90 est comparable avec

30 ce qui se passe pour le récepteur aux œstrogènes. En fait, six

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L2 RUN STATEMENT CREATED

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CANCER

L4 26402 S L2 AND (TUMOR# OR TUMOUR# OR ENDOTHELI? OR CANCER? OR
MALIGNA

L5 630 S L2 AND ENDOTHELI?

L6 541 S L5 AND (TUMOR# OR TUMOUR# OR CANCER? OR MALIGNAN?)

L7 331 S L6 AND (DETECT? OR DIAGNOS? OR IMAG? OR TARGET? OR DIRECT?)

L8 22 S L7 AND SQL<100

L9 86 S L7 AND SQL<200

L10 64 S L9 NOT L8

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L12 69 S L7 NOT (EIGHTY(W)SEVEN)/TI

L13 177 S L2 AND PY<2000

L14 59 S L13 AND PY<1999

L15 118 S L13 NOT L14

L16 12 S L15 AND SQL<51

RUN GETSEQ

L17 RUN STATEMENT CREATED

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MALIGN

L19 664 S L17 AND ENDOTHELI?

L20 565 S L19 AND (TUMOR# OR TUMOUR# OR CANCER? OR MALIGNAN?)

L21 2 S L20 AND PY<2001

L22 5673 S L17 AND SQL<51

L23 9 S L22 AND PY<1999

L24 32 S L22 AND PY<2001

L25 27 S L24 NOT L13

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L27	L26 and (cancer\$1 or malignan\$4 or tumor\$1 or tumour\$1 or neoplas\$3)	262	L27
L26	L25 and (endothelial adj cell\$)	393	L26
L25	L23 and (target\$3 or deliver\$3 or bind\$3)	21251	L25
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L23	peptide\$1 or oligopeptide\$1 or polypeptide\$1	71795	L23

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L20	l11 and l16	2	L20
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L17	L16 and @ad<20000316	48	L17
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L15	L2 with (peptide\$1 or oligopeptide\$1 or polypeptide\$1)	273	L15
L14	L13 and @ad<20000316	359	L14
L13	L2 with (cancer\$1 or malignan\$4 or tumor\$1 or tumour\$1 or neoplas\$3)	378	L13
L12	L11 and l5	3	L12
L11	l9 with l10	1064	L11
L10	(amino adj acid\$1) with hydrophobic	6142	L10
L9	(amino adj acid\$1) with (basic or positive\$2)	11618	L9
L8	L7 or l6	93	L8
L7	L5 and @prad<20000316	14	L7
L6	L5 and @ad<20000316	93	L6
L5	L3 with (cancer\$1 or malignan\$4 or tumor\$1 or tumour\$1 or neoplas\$3)	96	L5
L4	L3 and @ad<20000316	643	L4
L3	L2 with (peptide\$1 or oligopeptide\$1 or polypeptide\$1 or protein\$1)	674	L3

L3	L2 with (peptides\$1 or ongopeptides\$1 or polypeptides\$1 or proteins\$1)	0 / 4	L3
L2	L1 with (target\$3 or deliver\$3 or bind\$3)	2407	L2
L1	endothelial adj cell\$1	10752	L1

END OF SEARCH HISTORY

L16 ANSWER 9 OF 12 DGENE (C) 2002 THOMSON DERWENT
 ACCESSION NUMBER: ABB07076 Peptide DGENE
 TITLE: Protein having extensive virus infection inhibition
 activity,
 gene and strains thereof -
 INVENTOR: Kim Y S; Park S C; Oh S G; Cho J W; Chung C H
 PATENT ASSIGNEE: (KOKU-N)KOREA KUMHO PETROCHEMICAL CO LTD.
 PATENT INFO: KR 99055909 A 19990715 8p
 APPLICATION INFO: KR 1997-75882 19971229
 PRIORITY INFO: KR 1997-75882 19971229
 DOCUMENT TYPE: Patent
 LANGUAGE: Korean
 OTHER SOURCE: 2000-496266 [44]
 AN ABB07076 Peptide DGENE
 AA 0 A; 0 R; 0 N; 0 D; 0 B; 1 C; 1 Q; 0 E; 0 Z; 3 G; 0 H; 4 I;
 2
 L; 3 K; 4 M; 0 F; 0 P; 0 S; 3 T; 3 W; 0 Y; 4 V; 0 Others
 SQL 28
 SEQ
 1 mkkvlgggtw vwwcmimlil mittvvkq
 ===
 HITS AT: 2-4

102(a) 1, 2
 103(a) 7

L16 ANSWER 11 OF 12 DGENE (C) 2002 THOMSON DERWENT
ACCESSION NUMBER: ABB07048 Peptide DGENE
TITLE: Antivirus protein of amaranthin 1 and amaranthin 2 from
Amaranthus viridis and DNA coding the same -
INVENTOR: Kim Y S; Park S C; Oh S G; Lee H S; Cho J W; Chung C H
PATENT ASSIGNEE: (KOKU-N) KOREA KUMHO PETROCHEMICAL CO LTD.
PATENT INFO: KR 99055910 A 19990715 10p
APPLICATION INFO: KR 1997-75883 19971229
PRIORITY INFO: KR 1997-75883 19971229
DOCUMENT TYPE: Patent
LANGUAGE: Korean
OTHER SOURCE: 2000-496267 [44]
AN ABB07048 Peptide DGENE
AA 2 A; 0 R; 2 N; 0 D; 0 B; 1 C; 1 Q; 0 E; 0 Z; 0 G; 0 H; 4 I;
3
L; 3 K; 2 M; 0 F; 0 P; 1 S; 4 T; 1 W; 1 Y; 3 V; 0 Others
SQL 28
SEQ
1 mkmkkitnlv yilvaittsv ilqwtcna
===
HITS AT: 4-6

102 (a) 1, 2
103 (a) 4

L16 ANSWER 10 OF 12 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB07049 Peptide DGENE

TITLE: Antivirus protein of amaranthin 1 and amaranthin 2 from
Amaranthus viridis and DNA coding the same -

INVENTOR: Kim Y S; Park S C; Oh S G; Lee H S; Cho J W; Chung C H

PATENT ASSIGNEE: (KOKU-N) KOREA KUMHO PETROCHEMICAL CO LTD.

PATENT INFO: KR 99055910 A 19990715 10p

APPLICATION INFO: KR 1997-75883 19971229

PRIORITY INFO: KR 1997-75883 19971229

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 2000-496267 [44]

AN ABB07049 Peptide DGENE

AA 2 A; 0 R; 2 N; 0 D; 0 B; 1 C; 1 Q; 0 E; 0 Z; 0 G; 0 H; 4 I;
2

L; 3 K; 2 M; 1 F; 0 P; 1 S; 4 T; 1 W; 1 Y; 3 V; 0 Others

SQL 28

SEQ

1 mkmkkitnlv yilvaittsv ifqwtcna

===

HITS AT: 4-6

L16 ANSWER 12 OF 12 DGENE (C) 2002 THOMSON DERWENT
 ACCESSION NUMBER: AAU78259 Peptide DGENE
 TITLE: Fab region gene of mouse agglutination monoclonal antibody
 against human blood cell and base sequence thereof -
 INVENTOR: Lim S Y; Cho S H; Lee Y I; Bong Y S
 PATENT ASSIGNEE: (KOAD)KOREA ADV INST SCI & TECHNOLOGY.
 PATENT INFO: **KR 99048543 A 19990705** 9p
 APPLICATION INFO: KR 1997-67281 19971210
 PRIORITY INFO: KR 1997-67281 19971210
 DOCUMENT TYPE: Patent
 LANGUAGE: Korean
 OTHER SOURCE: 2000-449017 [39]
 AN AAU78259 Peptide DGENE
 AA 0 A; 1 R; 2 N; 0 D; 0 B; 1 C; 0 Q; 0 E; 0 Z; 2 G; 0 H; 1 I;
 0
 L; 2 K; 0 M; 1 F; 0 P; 1 S; 2 T; 0 W; 3 Y; 0 V; 0 Others
 SQL 16
 SEQ
 1 yiscyngtty nrkfkq
 ===
 HITS AT: 12-14

102 (a) 1 2
 103 (a) 4

L14 ANSWER 44 OF 59 DGENE (C) 2002 THOMSON DERWENT
 ACCESSION NUMBER: ABB05960 Protein DGENE
 TITLE: Recombinant microorganism expressing granulocyte colony
 stimulating factor and process for preparing the recombinant
 protein therefrom -
 INVENTOR: Sohn Y D; Lee E G; Kim S H; Park D H
 PATENT ASSIGNEE: (MOKA-N)MOKAM LIFE SCI RES CENT.
 PATENT INFO: **KR 98077885 A 19981116** **16p**
 APPLICATION INFO: KR 1997-15210 19970423
 PRIORITY INFO: KR 1997-15210 19970423
 DOCUMENT TYPE: Patent
 LANGUAGE: Korean
 OTHER SOURCE: 2000-035728 [03]
 AN ABB05960 Protein DGENE
 AA 21 A; 5 R; 0 N; 4 D; 0 B; 5 C; 18 Q; 10 E; 0 Z; 14 G; 5 H; 4 I;
 34 L; 4 K; 3 M; 6 F; 13 P; 14 S; 8 T; 3 W; 3 Y; 8 V; 0 Others
 SQL 182
 SEQ
 1 alwtvqeatz lgpasllpqs flkcleqvr kiqgdgaalq eklcatyklc
 = ==
 51 hpeelvllgh slgipwapls scpsqalqla gclsqlhsgl flyqgllqal
 101 egispelgpt ldtlqladvad fattiwqqme elgmapalqp tqgampafas
 151 afqrraggvl vashlqsfle vsyrvlrhla qp
 ===
 HITS AT: 30-32; 177-179

102 (b) 1, 2
 103 (a) 4

L14 ANSWER 42 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU77773 Peptide DGENE

TITLE: Process for preparing novel calcitonin -

INVENTOR: Choi C Y; Choi Y H

PATENT ASSIGNEE: (GREC)KOREA GREEN CROSS CORP.

PATENT INFO: KR 98047664 A 19980915

14p

APPLICATION INFO: KR 1996-66177 19961216

PRIORITY INFO: KR 1996-66177 19961216

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 1999-477713 [40]

AN AAU77773 Peptide DGENE

AA 0 A; 0 R; 2 N; 1 D; 0 B; 2 C; 2 Q; 0 E; 0 Z; 4 G; 1 H; 0 I;
3

L; 2 K; 1 M; 1 F; 2 P; 2 S; 7 T; 0 W; 2 Y; 0 V; 0 Others

SQL 32

SEQ

1 cgnlstcm1g tytqdfhklq typknttgsg tp

===

HITS AT: 17-19

102(b) 1, 2

L14 ANSWER 42 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU77773 Peptide DGENE

TITLE: Process for preparing novel calcitonin -

INVENTOR: Choi C Y; Choi Y H

PATENT ASSIGNEE: (GREC)KOREA GREEN CROSS CORP.

PATENT INFO: KR 98047664 A 19980915

14p

APPLICATION INFO: KR 1996-66177 19961216

PRIORITY INFO: KR 1996-66177 19961216

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 1999-477713 [40]

AN AAU77773 Peptide DGENE

AA 0 A; 0 R; 2 N; 1 D; 0 B; 2 C; 2 Q; 0 E; 0 Z; 4 G; 1 H; 0 I;
3

L; 2 K; 1 M; 1 F; 2 P; 2 S; 7 T; 0 W; 2 Y; 0 V; 0 Others

SQL 32

SEQ

1 cgnlstcm1g tytqdfhklq typknttgsg tp

===

HITS AT: 17-19

L14 ANSWER 45 OF 59 DGENE (C) 2002 THOMSON DERWENT
 ACCESSION NUMBER: ABB05959 Peptide DGENE
 TITLE: New mutant strains of rifampin-tolerant Mycobacterium leprae
 and its nucleotide sequence -
 INVENTOR: Suh J W; Chae G T; Kim S O
 PATENT ASSIGNEE: (SUHJ-I)SUH J W.
 (CHAE-I) CHAE G T.
 PATENT INFO: **KR 98068824 A 19981026** **10p**
 APPLICATION INFO: KR 1997-5613 19970224
 PRIORITY INFO: KR 1997-5613 19970224
 DOCUMENT TYPE: Patent
 LANGUAGE: Korean
 OTHER SOURCE: 1999-607936 [52]
 AN ABB05959 Peptide DGENE
 AA 2 A; 4 R; 2 N; 1 D; 0 B; 0 C; 2 Q; 1 E; 0 Z; 5 G; 1 H; 0 I;
 7
 L; 1 K; 1 M; 1 F; 2 P; 4 S; 1 T; 0 W; 0 Y; 0 V; 0 Others
 SQL 35
 SEQ
 1 lsqfmdqnnp lsglthkrll salpggglr eragl
 ===
 HITS AT: 18-20

102 (b) 1, 2

L14 ANSWER 30 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU77273 peptide DGENE

TITLE: Type IV collagenase activity inhibiting synthetic peptide
derived from tissue inhibitor of metalloproteinase-2

(TIMP-2)

- No Abstract

INVENTOR: Kim M Y; Kim S G; Lee J P; Koh J P; Lee D S; Lee J Y; Lee H
S

PATENT ASSIGNEE: (HANI-N)HANIL SYNTHETIC FIBER CO LTD.

PATENT INFO: KR 98073833 A 19981105

20p

APPLICATION INFO: KR 1997-9387 19970319

PRIORITY INFO: KR 1997-9387 19970319

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 2000-010130 [01]

AN AAU77273 peptide DGENE

AA 1 A; 1 R; 1 N; 1 D; 0 B; 1 C; 2 Q; 0 E; 0 Z; 1 G; 1 H; 0 I;
2

L; 2 K; 1 M; 0 F; 0 P; 2 S; 3 T; 0 W; 1 Y; 0 V; 0 Others

SQL 20

SEQ

1 dtlstdqkks lnhryqmgca

===

HITS AT: 13-15

102 (6) 1, 2

L14 ANSWER 29 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU77275 peptide DGENE

TITLE: Type IV collagenase activity inhibiting synthetic peptide
derived from tissue inhibitor of metalloproteinase-2

(TIMP-2)

- No Abstract

INVENTOR: Kim M Y; Kim S G; Lee J P; Koh J P; Lee D S; Lee J Y; Lee H
S

PATENT ASSIGNEE: (HANI-N)HANIL SYNTHETIC FIBER CO LTD.

PATENT INFO: KR 98073833 A 19981105

20p

APPLICATION INFO: KR 1997-9387 19970319

PRIORITY INFO: KR 1997-9387 19970319

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 2000-010130 [01]

AN AAU77275 peptide DGENE

AA 3 A; 1 R; 2 N; 1 D; 0 B; 2 C; 4 Q; 1 E; 0 Z; 1 G; 3 H; 0 I;
2

L; 2 K; 1 M; 1 F; 1 P; 2 S; 3 T; 0 W; 1 Y; 1 V; 0 Others

SQL 32

SEQ

1 vhpqqafcne hadtlsttqk kslnhryqmg ca

===

HITS AT: 25-27

L14 ANSWER 28 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU77280 peptide DGENE

TITLE: Type IV collagenase activity inhibiting synthetic peptide
derived from tissue inhibitor of metalloproteinase-2

(TIMP-2)

- No Abstract

INVENTOR: Kim M Y; Kim S G; Lee J P; Koh J P; Lee D S; Lee J Y; Lee H
S

PATENT ASSIGNEE: (HANI-N)HANIL SYNTHETIC FIBER CO LTD.

PATENT INFO: KR 98073833 A 19981105

20p

APPLICATION INFO: KR 1997-9387 19970319

PRIORITY INFO: KR 1997-9387 19970319

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 2000-010130 [01]

AN AAU77280 peptide DGENE

AA 1 A; 1 R; 1 N; 0 D; 0 B; 1 C; 1 Q; 0 E; 0 Z; 1 G; 1 H; 0 I;
1

L; 0 K; 1 M; 0 F; 0 P; 0 S; 0 T; 0 W; 1 Y; 0 V; 0 Others

SQL 10

SEQ

1 lnhryqmgca

===

HITS AT: 3-5

L14 ANSWER 27 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU77281 peptide DGENE

TITLE: Type IV collagenase activity inhibiting synthetic peptide
derived from tissue inhibitor of metalloproteinase-2

(TIMP-2)

- No Abstract

INVENTOR: Kim M Y; Kim S G; Lee J P; Koh J P; Lee D S; Lee J Y; Lee H
S

PATENT ASSIGNEE: (HANI-N)HANIL SYNTHETIC FIBER CO LTD.

PATENT INFO: KR 98073833 A 19981105

20p

APPLICATION INFO: KR 1997-9387 19970319

PRIORITY INFO: KR 1997-9387 19970319

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 2000-010130 [01]

AN AAU77281 peptide DGENE

AA 1 A; 1 R; 1 N; 0 D; 0 B; 2 C; 1 Q; 0 E; 0 Z; 1 G; 1 H; 0 I;
1

L; 1 K; 1 M; 0 F; 0 P; 0 S; 0 T; 0 W; 1 Y; 0 V; 0 Others

SQL 12

SEQ

1 kclnhryqmg ca

===

HITS AT: 5-7

L14 ANSWER 14 OF 59 DGENE (C) 2002 THOMSON DERWENT
ACCESSION NUMBER: ABB08914 peptide DGENE
TITLE: Use of beta-sheet forming amino acid leader sequence for the
production of proteins -
INVENTOR: Shin H; Jang S; Kim D; Kang S
PATENT ASSIGNEE: (HANI-N)HANIL SYNTHETIC FIBER CO LTD.
PATENT INFO: **KR 133475 B1 19980421** **14p**
APPLICATION INFO: KR 1994-7018 19940404
PRIORITY INFO: KR 1994-7018 19940404
DOCUMENT TYPE: Patent
LANGUAGE: Korean
OTHER SOURCE: 1999-617508 [53]
AN ABB08914 peptide DGENE
AA 0 A; 1 R; 2 N; 0 D; 0 B; 2 C; 2 Q; 1 E; 0 Z; 4 G; 1 H; 0 I;
5
L; 2 K; 0 M; 0 F; 2 P; 4 S; 5 T; 0 W; 1 Y; 1 V; 0 Others
SQL 33
SEQ
1 csnlstcvlg klsqelhklq typrnttgsg tpg
===
HITS AT: 17-19

102(b) 1, 2

L14 ANSWER 3 OF 59 DGENE (C) 2002 THOMSON DERWENT
ACCESSION NUMBER: ABB97979 Peptide DGENE
TITLE: Genetic engineering synthesis method of horseshoe crab
extract as anti-fungus polypeptide -
INVENTOR: Zhang C; Fan Y
PATENT ASSIGNEE: (BIOL-N) BIOLOGICAL TECHNOLOGY RES CENT CHINESE.
PATENT INFO: CN 1182135 A 19980520 20p
APPLICATION INFO: CN 1996-120608 19961107
PRIORITY INFO: CN 1996-120608 19961107
DOCUMENT TYPE: Patent
LANGUAGE: Chinese
OTHER SOURCE: 2002-395145 [43]
AN ABB97979 Peptide DGENE
AA 0 A; 6 R; 0 N; 0 D; 0 B; 4 C; 0 Q; 0 E; 0 Z; 1 G; 0 H; 0 I;
0
L; 1 K; 0 M; 2 F; 0 P; 0 S; 0 T; 1 W; 2 Y; 1 V; 0 Others
SQL 18
SEQ
1 rrwcfrcvyr gfcyrkcr
===
HITS AT: 1-3

102 (b) 1, 2

103 (a) 4

L14 ANSWER 2 OF 59 DGENE (C) 2002 THOMSON DERWENT
ACCESSION NUMBER: ABB97980 Peptide DGENE
TITLE: Genetic engineering synthesis method of horseshoe crab
extract as anti-fungus polypeptide -
INVENTOR: Zhang C; Fan Y
PATENT ASSIGNEE: (BIOL-N) BIOLOGICAL TECHNOLOGY RES CENT CHINESE.
PATENT INFO: CN 1182135 A 19980520 20p
APPLICATION INFO: CN 1996-120608 19961107
PRIORITY INFO: CN 1996-120608 19961107
DOCUMENT TYPE: Patent
LANGUAGE: Chinese
OTHER SOURCE: 2002-395145 [43]
AN ABB97980 Peptide DGENE
AA 0 A; 5 R; 0 N; 0 D; 0 B; 4 C; 0 Q; 0 E; 0 Z; 1 G; 0 H; 0 I;
0
L; 2 K; 0 M; 2 F; 0 P; 0 S; 0 T; 1 W; 2 Y; 1 V; 0 Others
SQL 18
SEQ
1 rrwcfrcvyk gfcyrkcr
===
HITS AT: 1-3

L14 ANSWER 51 OF 59 DGENE (C) 2002 THOMSON DERWENT
 ACCESSION NUMBER: AAM49057 protein DGENE
 TITLE: Preparing calcitonin gene related peptide (CGRP) for
 reducing blood pressure, comprises chemical synthesization -
 INVENTOR: Zheng Z; Yan W; Sun L
 PATENT ASSIGNEE: (UYFU-N)UNIV FUDAN.
 PATENT INFO: CN 1175586 A 19980311 6p
 APPLICATION INFO: CN 1997-106570 19970819
 PRIORITY INFO: CN 1997-106570 19970819
 DOCUMENT TYPE: Patent
 LANGUAGE: Chinese
 OTHER SOURCE: 2002-075830 [11]
 AN AAM49057 protein DGENE
 AA 4 A; 2 R; 3 N; 0 D; 0 B; 2 C; 0 Q; 0 E; 0 Z; 4 G; 1 H; 0 I;
 3 L; 2 K; 1 M; 2 F; 1 P; 4 S; 4 T; 0 W; 0 Y; 4 V; 0 Others
 SQL 37
 SEQ
 1 acntatcvth rlagllsrsg gmvksnfvpt nvgscaf
 = ==
 HITS AT: 10-12

102(b) 1, 2

103(a) 3

L14 ANSWER 52 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAM49056 protein DGENE

TITLE: Preparing calcitonin gene related peptide (CGRP) for
reducing

blood pressure, comprises chemical synthesization -

INVENTOR: Zheng Z; Yan W; Sun L

PATENT ASSIGNEE: (UYFU-N)UNIV FUDAN.

PATENT INFO: CN 1175586 A 19980311

6p

APPLICATION INFO: CN 1997-106570 19970819

PRIORITY INFO: CN 1997-106570 19970819

DOCUMENT TYPE: Patent

LANGUAGE: Chinese

OTHER SOURCE: 2002-075830 [11]

AN AAM49056 protein DGENE

AA 4 A; 2 R; 3 N; 1 D; 0 B; 2 C; 0 Q; 0 E; 0 Z; 4 G; 1 H; 0 I;
3

L; 2 K; 0 M; 2 F; 1 P; 3 S; 4 T; 0 W; 0 Y; 5 V; 0 Others

SQL 37

SEQ

1 acdtatcvth rlagllsrsg gvvknnfvpt nvgscaf
= ==

HITS AT: 10-12

L12 ANSWER 11 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB81649 Protein DGENE

TITLE: Novel apoptosis inducing molecule II polypeptide useful for treating lymphadenopathy, autoimmune disease, graft versus host disease and to inhibit neoplasia such as **tumor** cell growth -

INVENTOR: Ebner R; Yu G; Ruben S M; Ullrich S

PATENT ASSIGNEE: (HUMA-N)HUMAN GENOME SCI INC.

PATENT INFO: US 2002064869 A1 20020530

79p

APPLICATION INFO: US 1998-27287 19980220

PRIORITY INFO: US 1996-13923P 19960322

US 1996-30157P 19961031

US 1997-822953 19970321

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-556723 [59]

AN ABB81649 Protein DGENE

AA 20 A; 27 R; 17 N; 21 D; 0 B; 30 C; 15 Q; 30 E; 0 Z; 30 G; 10 H; 13 I; 58 L; 20 K; 5 M; 14 F; 37 P; 36 S; 31 T; 5 W; 13 Y; 23 V; 0 Others

SQL 455

SEQ

1 mglstvpdll lplvllellv giypsgvigl vphlgdrekr dsvcpggkyi
51 hpqnnsicct kchkgtylyn dcpgggqtd crecesgsft asenhlrhcl
101 scskcrkemg qveissctvd rdtvcgcrkn qyrhywsenl fqcfnclcl

===

151 ngtvhlscqe kqntvctcha gfflrenecv scsnckksle ctklclpqi
201 nvkgtedsgt tvllplviff glclslffi glmyryqrwk sklysivcgk
251 stpekegele gtttkplapn psfsptpgft ptlgfsvpvs stftsssty
301 pgdcpnfap rrevappyg adpilatala sdipnplqk wedsahkpqs
351 ldtddpatly avvenvpplr wkefvrrlgl sdheidrlcl qngrclreaq

===

401 ysmlatwrrr tprreatlel lgrvlrdmdl lgcledieea lcgpaalppa
451 psllr

HITS AT: 133-135; 376-378

*Larger
sequences*

L12 ANSWER 10 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB81652 Protein DGENE

TITLE: Novel apoptosis inducing molecule II polypeptide useful for treating lymphadenopathy, autoimmune disease, graft versus host disease and to inhibit neoplasia such as **tumor** cell growth -

INVENTOR: Ebner R; Yu G; Ruben S M; Ullrich S

PATENT ASSIGNEE: (HUMA-N)HUMAN GENOME SCI INC.

PATENT INFO: US 2002064869 A1 20020530

79p

APPLICATION INFO: US 1998-27287 19980220

PRIORITY INFO: US 1996-13923P 19960322

US 1996-30157P 19961031

US 1997-822953 19970321

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-556723 [59]

AN ABB81652 Protein DGENE

AA 9 A; 11 R; 10 N; 4 D; 0 B; 4 C; 12 Q; 13 E; 0 Z; 19 G; 7 H; 4 I;
35 L; 16 K; 12 M; 11 F; 39 P; 26 S; 12 T; 4 W; 14 Y; 19 V; 0 Others

SQL 281

SEQ

1 mqqpfnyppp qiywvdssas spwappgtvl pcptsvprp gqrrpppppp

51 ppplpppppp pplpplpplp lkkrgnhstg lcillvmffmv lvalvglglg

101 mfqlfhlqke laelrestsq mhtasslekq ighpspppek kelrkvahlt

===

151 gksnsrsmpl ewedtygivl lsgvkykkgg lvinetglyf vyskvyfrgq

201 scnnlplshk vymrnskypq dlvmmegkmm sycttgqmw rssylgavfn

== =

251 ltsadhlyvn vselslvnfe esqtffglyk l

HITS AT: 144-146; 209-211

L12 ANSWER 22 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU99098 Protein DGENE

TITLE: Use of modulator of activity of novel glycosyl transferase proteins, 47169/33935 proteins, for making a medicament for modulating ability of cell to affect glycosylation state of lipid or polypeptide **target** in cell -

INVENTOR: Meyers R; Williamson M

PATENT ASSIGNEE: (MILL-N)MILLENNIUM PHARM INC.

PATENT INFO: WO 2002040657 A2 20020523 153p

APPLICATION INFO: WO 2001-US47575 20011120

PRIORITY INFO: US 2000-249939P 20001120

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-508326 [54]

AN AAU99098 Protein DGENE

AA 41 A; 48 R; 24 N; 38 D; 0 B; 13 C; 18 Q; 35 E; 0 Z; 42 G; 17 H; 27 I;
50 L; 35 K; 16 M; 23 F; 39 P; 36 S; 25 T; 17 W; 22 Y; 37 V; 0 Others

SQL 603

SEQ

```
1 mrrkekrllq avalvlaalv llpnvglwal yrerqpdgtp ggsgaavapa
===
51 agqgshsrqk ktfflgdgqk lkdwhdkeai rrdaqrvng eggrpypmtd
101 aervdqayre ngfnivy sdk islnrslpdi rhpcnnskry letlpntsii
===
151 ipfhnegwss llrtvhsvln rspplvaei vlvdffsdre hlkkp ledym
201 alfpsvrilr tkkreglirt rmlgasvatg dvtfldshc eanvnwlppl
251 ldriarnrkt ivcpmidvid hddfryetqa gdamrgafdw emyykripip
===
301 pelqkadpsd pfespvmagg lfavdrkwfw elggydpgle iwggeqyeis
===
351 fkvwmcggrm edipcsrvgh iyrkyvpykv pagvslarnl krvaevwmde
===
401 yaeyiyqrrp eyrhlsagdv avqkklrssl ncksfkwfmt kiawdlpkfy
===
451 ppveppaaaw geirnvgtgl cadtkhgalg splrlegcvr grgeaawnm
501 qvftftwred irpgdpqhtk kfcfdaisht spvtlydchs mkgnqlwkyr
= ==
551 kdktylhpvs gscmdcsesd hrifmntcnp ssltqqwlfe htnstvlekf
===
```

601 nrn

HITS AT: 6-8; 138-140; 295-297; 326-328; 373-375; 391-393; 413-415; 424-426;
520-522; 571-573

L12 ANSWER 20 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAE24240 Protein DGENE

TITLE: New human carboxypeptidase, scramblase, and protocadherin protein and polynucleotides for identifying modulators for use in **diagnosing**, treating diabetes mellitus, atherosclerosis, **cancer**, Alzheimer's disease -

INVENTOR: Meyers R A; Curtis R A J; Kapeller-libermann R

PATENT ASSIGNEE: (MILL-N)MILLENNIUM PHARM INC.

PATENT INFO: WO 2002033088 A2 20020425 170p

APPLICATION INFO: WO 2001-US46717 20011022

PRIORITY INFO: US 2000-241989P 20001020

US 2000-242324P 20001020

US 2000-242518P 20001023

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-471400 [50]

AN AAE24240 Protein DGENE

AA 37 A; 34 R; 18 N; 16 D; 0 B; 13 C; 14 Q; 18 E; 0 Z; 27 G; 17 H; 26 I;

38 L; 21 K; 9 M; 16 F; 20 P; 38 S; 26 T; 11 W; 25 Y; 20 V; 0 Others

SQL 444

SEQ

1 mdsltcpqsl vcvglriswl feksvfsfta cspwptcgrr rvlslsvkti

= ==

51 nvalshvlmy flparaaara athyraraat hyraraathy raraathyra

101 raaaragihs qghlihcqrg pefsnllcgr mkplletlyl lgmlvpgglg

151 ydrslaqrq eivdksvspw sletysyniy hpmgeiyewm reisekykev

201 vtqhflgvty ethpiyylki sqpsgnpkki iwmdcgihar ewiapafcqw

===

251 fvkeilqnhk dnsrirkllr nldfyvlpvl nidgyiytw tdlrwrksrs

===

301 phnngtcfgt dlrrnfnasw csigasrncq dqtfcgtgpv sepetkavas

351 fieskkddil cfltmhsygg liltpygytk nkssnhpemi qvgqkaanal

401 kakygtnyrv gssadilyas sgssrdward igipfsytfe lrdr

HITS AT: 40-42; 228-230; 266-268

L12 ANSWER 36 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB76177 Protein DGENE

TITLE: Composition for treating or preventing pathological conditions in which **endothelial** cells are involved or affected e.g. diabetes, inflammation, psoriasis, has Raf protein, polynucleotide, or modulators of the protein -

INVENTOR: Hatzopoulos A; Hautmann M; Herbst M; Geishauser A; Schoch J

PATENT ASSIGNEE: (GSFU-N)GSF FORSCHUNGSZENTRUM UMWELT & GESUNDHEI.

PATENT INFO: WO 2002026246 A2 20020404 64p

APPLICATION INFO: WO 2001-EP11282 20010928

PRIORITY INFO: EP 2000-121490 20000929

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-402033 [43]

AN ABB76177 Protein DGENE

AA 39 A; 42 R; 18 N; 29 D; 0 B; 14 C; 32 Q; 26 E; 0 Z; 40 G; 20 H; 22 I;
55 L; 30 K; 16 M; 26 F; 43 P; 53 S; 34 T; 6 W; 16 Y; 44 V; 1 Others

SQL 606

SEQ

1 mepprgppan gaepsravgt vkvylpnkqr tvvtvrdgms vydsldkalk
51 vrglnqdcv vyrlikgrkt vtawdtaiap ldgeelivev ledvpltmhn
101 fvrktffsla fcdclckflf hgfrqcqcy kfhqhcsskv ptcvcdmstn
151 rqqfyhsvqd lsggsrqhea psnrplnell tpqgpsprtq hcdpehfpfp
201 apanaplqri rststpnvbm vsttapmdsn liqltggsfs tdaagsrggs
251 dgtprgspsp asvssgrksp hskspaeqre rksladdkkk vknlgryxsg

== =

301 yywevppsev qllkrightgs fgtvfrgrwh gdvavkvlkv sqptaeqaa

===

351 fknemqvlrk trhvnillfm gfmtrpgfai itqwcegssl yhhlhvadtr

===

401 fdmvqlidva rqtatgmdyl hakniihrdl ksnniflgeg ltvkigdfgl

451 atvktwsga qpleqpsgsv lwmaaevirm qdpnpysfqs dvyaygvvly

501 elmtgslpys higcrdqiif mvgrgylspd lskissncpk amrllsdcl

===

551 kfgreerplf pqilatliell qrsllpkiers asepslhrtq adelpaclls

601 aarlvp

HITS AT: 289-291; 314-316; 362-364; 392-394; 543-545

L12 ANSWER 33 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB80018 Protein DGENE

TITLE: New isolated nucleic acids encoding tyrosine kinase containing Immunoglobulin and Epidermal Growth Factor (TIE) ligand NL2, for **diagnosing** and treating wounds, **cancer**, and ischaemia and promoting bone and muscle development -

INVENTOR: Goddard A; Godowski P J; Gurney A L

PATENT ASSIGNEE: (GETH)GENENTECH INC.

PATENT INFO: US 6372491 B1 20020416 43p

APPLICATION INFO: US 2000-511133 20000223

PRIORITY INFO: US 1997-59352P 19970919

US 1998-143707 19980828

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-392224 [42]

AN ABB80018 Protein DGENE

AA 35 A; 29 R; 11 N; 17 D; 0 B; 7 C; 36 Q; 19 E; 0 Z; 34 G; 18 H; 8 I;
47 L; 16 K; 7 M; 14 F; 23 P; 32 S; 18 T; 10 W; 5 Y; 19 V; 1 Others

SQL 406

SEQ

```
1 msgaptagaa lmlcaatavl lsaqggpvqs ksprfaswde mnvlahgllq
51 lgqglrehae rtrsqlsale rrlsacgsac qgtegstdlp lapesrvdpe
=====
101 vlhslqtqlk aqnsriqqfl hkvaqqqrhl ekqhlriqhl qsqfglldhk
=====
151 hldhevakpa rkrpemaq pvdpahnvsl lhrprdcqe lfqvgerqsg
=====
201 lfeiqpggsp pflvnckmts xggtviqrr hdgsvdfnpr weaykagfgd
251 phgefwwgle kvhsitgdrn srlavqlrdw dgnaellqfs vhlgedtay
301 slqltapvag qlgattvpps glsvpfstwd qdhnllrdkn caksllsggw
351 fgtcshsnln gqyfrsipqq rqlkkgifw ktwrgryypl qattmliqpm
401 aaeaas
```

HITS AT: 71-73; 121-123; 128-130; 150-152; 163-165; 182-184

L12 ANSWER 32 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB80019 Protein DGENE

TITLE: New isolated nucleic acids encoding tyrosine kinase containing Immunoglobulin and Epidermal Growth Factor (TIE) ligand NL2, for **diagnosing** and treating wounds, **cancer**, and ischaemia and promoting bone and muscle development -

INVENTOR: Goddard A; Godowski P J; Gurney A L

PATENT ASSIGNEE: (GETH)GENENTECH INC.

PATENT INFO: US 6372491 B1 20020416 43p

APPLICATION INFO: US 2000-511133 20000223

PRIORITY INFO: US 1997-59352P 19970919

US 1998-143707 19980828

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-392224 [42]

AN ABB80019 Protein DGENE

AA 20 A; 19 R; 11 N; 12 D; 0 B; 8 C; 9 Q; 17 E; 0 Z; 37 G; 10 H; 3 I;
33 L; 7 K; 5 M; 11 F; 22 P; 23 S; 9 T; 10 W; 10 Y; 12 V; 0 Others

SQL 288

SEQ

```
1 mdllwilpsl wllllggpac lktqehpscp gpreleaskv vllpscpgap
51 gspgekgapg pqgppgppgk mgpkgepgpr ncrellsqga tlgwyhlcl
101 pegralpvfc dmdtegggwl vfqrrqdgsv dffrswssyr agfgnqesef
151 wlgnenlhql tlqgnwelrv eledfngnrt fahyatfrll gevdhyqlal
201 gkfsegtagd slslhsggrp ttydadhdss nsncavivhg awwyascyrs
251 nlngryavse aaahkygidw asgrgvghpy rrvrmmlr
```

===

===

HITS AT: 264-266; 281-283

L12 ANSWER 31 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB80020 Protein DGENE

TITLE: New isolated nucleic acids encoding tyrosine kinase containing Immunoglobulin and Epidermal Growth Factor (TIE) ligand NL2, for **diagnosing** and treating wounds, **cancer**, and ischaemia and promoting bone and muscle development -

INVENTOR: Goddard A; Godowski P J; Gurney A L

PATENT ASSIGNEE: (GETH)GENENTECH INC.

PATENT INFO: US 6372491 B1 20020416 43p

APPLICATION INFO: US 2000-511133 20000223

PRIORITY INFO: US 1997-59352P 19970919

US 1998-143707 19980828

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-392224 [42]

AN ABB80020 Protein DGENE

AA 9 A; 19 R; 32 N; 22 D; 0 B; 4 C; 27 Q; 41 E; 0 Z; 23 G; 15 H; 31 I;
48 L; 38 K; 5 M; 18 F; 19 P; 38 S; 25 T; 9 W; 18 Y; 19 V; 0 Others

SQL 460

SEQ

1 mftiklllfi vplvissrid qdnssfdsls pepksrfaml ddvkilangl
51 lqlghglkdf vhktkgqind ifqklnifdq sfydslsqt eikeeekelr
101 rttyklqvkn eevknmslel nsklesllee killqqkvky leeqlnliq
151 nqpetpehpe vtslktfvek qdnsikdllq tvedqykqln qqhsqikeie
201 nqlrrtsiqe pteislsskp raprttflq lneirnvkhd gipaecttiy
251 nrgehtsgmy airpsnsqvf hvycdvisgs pwtliqhrid gsqnfnetwe
====

301 nykygfgrld gefwlgleki ysivkqsnyv lrieledwkd nkhyieysfy
====

351 lgnhetnytl hlvaitgnvp naipenkdlv fstwdhkakg hfncpegysg

401 gwwwdecge nnlngkynkp rakskperrr glswksqngr lysikstkml

451 ihptdsesfe

HITS AT: 287-289; 342-344

L12 ANSWER 45 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU79810 Protein DGENE

TITLE: New human matrix metalloproteinase and polynucleotides
useful

for **diagnosing** and treating atherosclerosis,
bacterial and viral infections, wound healing, chronic
injury, traumatic, ischaemia and psoriasis -

INVENTOR: Curtis R A J

PATENT ASSIGNEE: (MILL-N)MILLENNIUM PHARM INC.

PATENT INFO: WO 2002020739 A2 20020314 133p

APPLICATION INFO: WO 2001-US28260 20010910

PRIORITY INFO: US 2000-231136P 20000908

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-351776 [38]

AN AAU79810 Protein DGENE

AA 52 A; 50 R; 12 N; 27 D; 0 B; 5 C; 25 Q; 24 E; 0 Z; 47 G; 14 H; 11 I;
57 L; 18 K; 5 M; 28 F; 37 P; 28 S; 18 T; 19 W; 17 Y; 26 V; 0 Others

SQL 520

SEQ

1 mvarvglllr alqlllwghl daqpaerggq elrkeaeafk ekygylneqv
51 pkaptstrfs dairafqwvs qlpvsgvldr atlrqmrtrpr cgvttdtnsya
101 awaerisdfl arhrtkmrrk krfakqgnkw ykqhlsyrlv nwpehlpepa
===

151 vrgavraafq lwsnvsalef weapatgpad irltffqgdh ndglgnafdg
201 pggalahafk prrgeahfdq derwslsrrr grnlfvviah eightlglth
251 spapralmap yykrlgrdal lswddvlavq slygkplggs vavqlpgklf
===

301 tdfetwdsys pqgrrpetqg pkychssfda itvdrqqgly ifkgshfwev
351 aadgnvsepr plqerwvglp pniesaaavsl ndgdfyffkg grcwrfrgpk
401 pvwglpqlcr agglprhpda alffpplrrl ilfkgaryyv largglqvep
===

451 yyprslqdwg gipeevsgal prpdgsiiff rddrywrlldq aklqattsg
501 watelpwmgc whansgsalf

HITS AT: 121-123; 263-265; 428-430

L12 ANSWER 43 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB75939 Protein DGENE

TITLE: Novel recombinant protein termed angioquiescin useful for treating **tumor**, ulcer, comprises amino acid sequence corresponding to Kringle 1-5, secretory signal peptide and pre-activation peptide of mammalian plasminogen

INVENTOR: Cao Y

PATENT ASSIGNEE: (KARO-N)KAROLINSKA INNOVATIONS AB.

PATENT INFO: WO 2002020813 A2 20020314

63p

APPLICATION INFO: WO 2001-EP10090 20010831

PRIORITY INFO: US 2000-230893P 20000905

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-362251 [39]

AN ABB75939 Protein DGENE

AA 37 A; 42 R; 40 N; 36 D; 0 B; 48 C; 31 Q; 56 E; 0 Z; 62 G; 24 H; 21 I;
48 L; 49 K; 11 M; 21 F; 69 P; 56 S; 61 T; 19 W; 30 Y; 49 V; 0 Others

SQL 810

SEQ

1 mehkevvl11 llflksggqe plddyvntqg aslfsvttkq lgagsieeca
51 akceedeef crafqyhske qqcvmnenr kssiiirmrd vvlfeckvyl
===

101 secktgngkn yrgtmsktkn gitcqkwsst sphrprfspa thpsegleen
151 ycrnpdndpq gpwcyttde krydydile ceeecmhcs gnydgkiskt
===

201 msglecqawd sqspahgyi pskfpnknk knycrnpdre lrpwcfttdp
251 nkrwelcdip rcttppps gtyqclkgtg enyrgnvavt vsghcqhws
===

301 aqtpthnrt penfpcknld enycrnpdgk rapwchttns qvrweyckip
351 scdsspvste qlaptappel tpvvqdcyhg dgqsyrgtss ttttgkkcqs
401 wssmtphrhq ktpenypnag ltmnycrnpd adkgpwcftt dpsvrweygn
451 lkkcsgteas vvappvvll pdvetpseed cmfgngkgyr gkrattvtgt
501 pcqdwaapep hrhsiftpet npraglekny crnpdgdvvg pwcyttmprk
==

551 lydycdvpqc aapsfcdgkp qvepkckpgr vvggcvahph swpwqvsrlt
=

601 rfgmhfcggt lispewvlta ahcleksprp ssykvilgah qevnlephvq
651 eievslrfle ptrkdiallk lsspavitdk vipaclpspn yvvadrtecf
701 vtgwgetqgt fgagllkeaq lpvienkvcn ryeflngrvq stelcaghla
751 ggtdscqgds ggplvcfekd kyilqgvtsw glgcarpnkp gvyvrvsrfv
801 twiegvmrnn

HITS AT: 96-98; 171-173; 252-254; 549-551

L12 ANSWER 38 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB80688 Protein DGENE

TITLE: New canine angiostatin polypeptide, useful in treatment and
diagnosis of angiostatin-related disorders e.g.
cancer and rheumatoid arthritis and to screen for
modulating compounds useful to treat such disorders -

INVENTOR: Sheppard M G; Tong X

PATENT ASSIGNEE: (PFIZ) PFIZER PROD INC.

PATENT INFO: EP 1197550 A2 20020417

57p

APPLICATION INFO: EP 2001-307230 20010824

PRIORITY INFO: US 2000-228000 20000825

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-354188 [39]

AN ABB80688 Protein DGENE

AA 17 A; 23 R; 29 N; 18 D; 0 B; 30 C; 18 Q; 37 E; 0 Z; 27 G; 15 H; 11 I;
12 L; 28 K; 10 M; 12 F; 45 P; 36 S; 35 T; 12 W; 20 Y; 16 V; 0 Others

SQL 451

SEQ

1 crafqyhske qqcvmipens kssivfrmrdr vflfekriyl secktgngkt

===

51 yrgtmaktkn dvacqkwsdn sphkpnytp khplegleen ycrnpdnden

101 gpwcyttndp vrfdydni ceeecmhcs enyegkiskt ksglecqawn

151 sqtpahgyi pskfpsknk mnycrnpdge prpwcftmdp nkrwefcdip

===

201 rcttppppsg ptyqclkg rg esyrgkvs vt vsghcqhws eqtphkhnrt

251 penfpcknld enycrnpdge tapwcytt ns evrwehcqip scesspitte

301 yldapasvpp eqtpvvqecy hgngqsyr gt ssttitgrkc qswssmtphr

351 hektpehfpe agltmnycrn pdadkspwcy ttdpsvrwef cnlrkcldpe

401 asatnspavp qvpsgqepsa sdcmfngngk yrgkkattvm gipcqwaaq

451 e

HITS AT: 36-38; 192-194

L14 ANSWER 1 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB84401 Protein DGENE

TITLE: Isolated nucleic acid encoding a human kinase, useful for treating autoimmune disorders and malignancies -

INVENTOR: Roifman C M

PATENT ASSIGNEE: (HSCR-N)HSC RES & DEV LP.

PATENT INFO: CA 2203706 A 19981025

57p

APPLICATION INFO: CA 1997-2203706 19970425

PRIORITY INFO: CA 1997-2203706 19970425

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2000-024038 [03]

AN ABB84401 Protein DGENE

AA 51 A; 61 R; 68 N; 54 D; 0 B; 27 C; 46 Q; 86 E; 0 Z; 62 G; 33 H; 75 I;
108L; 78 K; 30 M; 53 F; 52 P; 71 S; 55 T; 14 W; 49 Y; 60 V; 0 Others

SQL 1133

SEQ

1 mgmacitmt megtstssiy qngdisgnan smkqidpvlq vylyhslgkp
51 eadyltfpsg eyvaeecia askacgitpv yhnmfalmse teriwyppnh
101 vfhidestrh nvlyrirfyf prwycsgsnr ayrhgisrga eapllddfv
151 sylfaqwrhd lvhgwikvpv tretqecclg tavlhmria kendqtplai
201 ynsisyktfl pkcirakiqd yhiltrkrir yrfrfiiqqf sqckatarnl
===

251 klkylinlet lqsafytekf evkepgsgps geeifatiii tgnggiqwsr
301 gkhkesetlt eqdlglycdf pniidvsikq anqegsnesr vvtihkqdgk
351 nleiielssl realsfvsli dgyyrltada hhylckevap pavleniqsn
===

401 chgpismvfa isklknagnq tglyvlrcsp kdfnkyfltf averenviey
451 khclitkn enynlsgtkk nfsslkdl n cyqmetvr sd niifqftkcc
501 ppkpdkdksnl lvfrtngv sd vptsptlqrp thmqmvmfhk irnedlifne
==

551 slgqgtftki fkgvrrevgd ygqlhetevl lkvl dkahrn ysesffeaas
601 mmsklshkhl vlnygvvcvg denimvqefv kfgsl dtylk knkncinilw
===

651 klevakqlaw amhfleentl ihgnvcakni qlireedrkt gnppfiklse
701 pgisitvlpk dilqeripwv ppecienpkn lnlatdkwsf gttlweicsg
751 gdkplsalds qrklqfyedr hqlpapkwa e lanlinncmd yepdfrpsfr
===

801 aiirdlnslf tpdyellten dmlpnmrnga lgfsgafedr dptqfeerhl
===

851 kflqqlgkgn fgsvemcryd plqdn tgevv avkklqhste ehldr ferei
===

901 eilkslqhdn ivkykgvcys agrnkl klim eylpygslrd ylqkhkerid
951 hikllqytsq ickgmvy lgt kryihrdlat rn ilvenenr vkigdfgltk
===

1001 vlpqdkeyyk vkepgespif wyapesltes kfsvasdvws fgvvlyelft
1051 yieksksppa efmr migndk qgqmivfhli ellknng rlp rpdgcpdeiy
1101 mimtecwnnn vnqrpsfrdl vlrvdqvr dn mag

HITS AT: 227-229; 234-236; 381-383; 539-541; 608-610; 762-764; 848-850;
883-885; 971-973

L14 ANSWER 50 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAM49503 Protein DGENE

TITLE: Gene clone of inhibitory factor for hyperplasia of inner
blood vessel cells in human body's real tumor, and its use
in

anti-tumor blood vessel regeneration -

INVENTOR: Xu G; Ren M; Xu L

PATENT ASSIGNEE: (XUGG-I)XU G.

PATENT INFO: CN 1177005 A 19980325

6p

APPLICATION INFO: CN 1997-107112 19970910

PRIORITY INFO: CN 1997-107112 19970910

DOCUMENT TYPE: Patent

LANGUAGE: Chinese

OTHER SOURCE: 2002-106746 [15]

AN AAM49503 Protein DGENE

AA 18 A; 15 R; 4 N; 8 D; 0 B; 4 C; 8 Q; 7 E; 0 Z; 16 G; 7 H; 6 I;
20 L; 5 K; 2 M; 10 F; 10 P; 20 S; 7 T; 4 W; 3 Y; 9 V; 0 Others

SQL 183

SEQ

1 hshrdfqpv l hlvalnspls ggmrgirgad fqcfqgarav glagtfracfl
51 ssrlqdlysi vrradraavp ivnlkdellf pswealfsgs egplkpgari
101 fsfdgkdvlr hptwpqksvw hgspnngrrl tesycetwrt eapsatgqas
===

151 sllggrllgq saaschhayi vlciensfmt ask

HITS AT: 128-130

L12 ANSWER 69 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAE18552 Protein DGENE

TITLE: Novel human ATPase polypeptide and nucleic acid molecules
for

diagnosing and treating cellular proliferative
disorders e.g. **cancer**, and brain, heart, bone or
liver disorders, viral diseases and identifying modulators -

INVENTOR: Meyers R; Williamson M

PATENT ASSIGNEE: (MILL-N)MILLENNIUM PHARM INC.

PATENT INFO: WO 2002006465 A2 20020124 124p

APPLICATION INFO: WO 2001-US22165 20010712

PRIORITY INFO: US 2000-218053P 20000713

US 2000-715479 20001117

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-171807 [22]

AN AAE18552 Protein DGENE

AA 74 A; 43 R; 20 N; 41 D; 0 B; 13 C; 27 Q; 47 E; 0 Z; 52 G; 20 H; 25 I;
60 L; 29 K; 14 M; 18 F; 46 P; 58 S; 26 T; 4 W; 17 Y; 31 V; 0 Others

SQL 665

SEQ

1 mevsgpeddp flsqlhqvqc pvcqqmmpaa hinshldrcl llhpaghaep
51 aagshrager akgpsppgak rrrlsessal kqpatptaae ssegegeegd
===

101 dggetesres ydapptpsga rlipdfpvar ssspgrkgsg krpaaaaaag
151 sasprswdea eaqeaaaavg dgdgdgdada dgeddpghwd adaaeaataf
201 gasgggrphp ralaeeeirg mlqgkpladt mrpdtlqdyf gqskavgqdt
251 llrsilletne ipsllilwgpp gcgkttlahi iasnskkhsi rfvtlsatna
301 ktndvrdivik qaqneksffk rktilfidei hrfnksqqdt flphvecgti
===

351 tligattenp sfqvnaalls rcrvivlekl pveamvtilm rainslgiHV
401 ldssrptdpl shssnssep amfiedkavd tlaylsdgda raglnglqla
451 vlarlssrkm fckksgqsys psrvlitend vkeglqrshi lydrageehy
===

501 ncisalhksm rgdqnasly wlarmlegge dplyvarrlv rfasedigla
===

551 dpsaltqava ayqgchfigm pecevllaqc vvyfarapks ievysaynnv
601 kacrlnhqgp lppvplhlrn aptrlmkdlg ygkgykynpm ysepvdqeyl
651 peelrgvdff kqrrc

HITS AT: 72-74; 331-333; 458-460; 537-539

L25 ANSWER 22 OF 27 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB76982 Peptide DGENE

TITLE: Novel inverted CCAAT box binding protein, and related nucleic

acids, antibodies and specific ligands, useful for treating and preventing cancer -

INVENTOR: Bronner C; Hopfner R; Mousli M; Jeltsch J; Lutz Y; Oudet P

PATENT ASSIGNEE: (ADER-N)ADEREGEM ASSOC DEV RECH EN GENETIQ.

PATENT INFO: WO 2000078949 A1 20001228 — 115p

APPLICATION INFO: WO 2000-FR1747 20000622

PRIORITY INFO: FR 1999-7935 19990622

DOCUMENT TYPE: Patent

LANGUAGE: French

OTHER SOURCE: 2001-091571 [10]

AN ABB76982 Peptide DGENE

AA 0 A; 3 R; 2 N; 3 D; 0 B; 3 C; 0 Q; 0 E; 0 Z; 1 G; 1 H; 0 I;
1

L; 3 K; 2 M; 0 F; 2 P; 3 S; 0 T; 0 W; 0 Y; 2 V; 0 Others

SQL 26

SEQ

1 mvdnprmrrks gpsckhckdd vnrlcs

===

HITS AT: 8-10

SEQ ID NO: 6

L12 ANSWER 68 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU75587 Protein DGENE

TITLE: A non-Goodpasture fragment of alpha3(IV)NC1 domain used in
detecting and treating disorders involving
angiogenesis -

INVENTOR: Kalluri R

PATENT ASSIGNEE: (BETH-N)BETH ISRAEL DEACONESS MEDICAL CENT.

PATENT INFO: WO 2001051523 A2 20010719 205p

APPLICATION INFO: WO 2001-US565 20010108

PRIORITY INFO: US 2000-479118 20000107

US 2000-543371 20000404

US 2000-625191 20000721

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-188037 [24]

AN AAU75587 Protein DGENE

AA 17 A; 13 R; 9 N; 5 D; 0 B; 12 C; 8 Q; 11 E; 0 Z; 17 G; 8 H; 11 I;
14 L; 5 K; 9 M; 11 F; 16 P; 25 S; 15 T; 4 W; 9 Y; 10 V; 0 Others

SQL 229

SEQ

1 svdhgflvtr hsqtiddpqc psgtkilyhg yslllyvqgne rahgqdlgta
51 gsclrkfstm pflfcnnnv cnfasrndys ywlstpepmp msmapitgen
===

101 irpfisrcav ceapamvmav hsqtiqippc psgwsslwig ysfvmhtsag

151 aegsggalas pgscleefrs apfiechgrg tcnyyanays fwlatierse

201 mfkktptstl kagelrthvs rcqvcmrrt

HITS AT: 55-57

L14 ANSWER 9 OF 59 DGENE (C) 2002 THOMSON DERWENT
 ACCESSION NUMBER: AAO17801 Protein DGENE
 TITLE: Pneumococcal surface protein SpsA - for use in producing vaccines
 INVENTOR: Chhatwal G S; Hammerschmidt S
 PATENT ASSIGNEE: (GBFB)GES BIOTECHNOLOGISCHE FORSCHUNG MBH.
 PATENT INFO: DE 19708537 A1 19980910 12p
 APPLICATION INFO: DE 1997-19708537 19970303
 PRIORITY INFO: DE 1997-19708537 19970303
 DOCUMENT TYPE: Patent
 LANGUAGE: German
 OTHER SOURCE: 1998-481924 [42]
 AN AAO17801 Protein DGENE
 AA 50 A; 14 R; 39 N; 20 D; 0 B; 0 C; 22 Q; 55 E; 0 Z; 35 G; 3 H; 12 I; 28 L; 57 K; 12 M; 5 F; 12 P; 44 S; 35 T; 19 W; 32 Y; 29 V; 0 Others
 SQL 523
 SEQ
 1 mfaskserkv hysirkfsig vasvvvaslv mgsvvhaten egstqaatfs
 ===
 51 nmanksqteq geinierdka ktavseykek kvseiyytkle rdrhkdtvdl
 = ==
 101 vnklqeikne ylnkivqsts kteiqglitt srskldeavs kykkapssss
 151 ssgsstkpea sdtakpnkpt elekkvaeae kkveeakkka kdqkeedyrn
 ===
 201 yptityktle leiaesdvev kkaelelvke eakeprneek vkqakakves
 251 eeteatrlek iktdrkkaee eakrkaaeed kvkekpaeqq aeedyarrse
 301 eeynrltqqq ppktekpaqp stpktgwkqe ngmwyfyntd gsmatgwlqn
 351 ngswyylnsn gamatgwlqn ngswyylnan gsmatgwlqn ngswyylnan
 401 gsmatgwlqy ngswyylnan gdmatgwlqy ngswyylnan gdmatgwlqy
 451 ngswyylnan gdmatgwkvd gdtwyyleas gamkasqwfk vsdkwyyvng
 501 sgalaavnttv dgygvnange wvn
 HITS AT: 8-10; 15-17; 80-82; 174-176; 181-183

L14 ANSWER 8 OF 59 DGENE (C) 2002 THOMSON DERWENT
 ACCESSION NUMBER: ABB09587 Protein DGENE
 TITLE: Gene related to rat's and human hypertension (HRG-1), useful
 for curing tumor and cardiovascular disease -
 INVENTOR: Tang J; Chen G
 PATENT ASSIGNEE: (UYBE-N)UNIV BEIJING MEDICAL.
 PATENT INFO: CN 1181420 A 19980513 7p
 APPLICATION INFO: CN 1997-121655 19971121
 PRIORITY INFO: CN 1997-121655 19971121
 DOCUMENT TYPE: Patent
 LANGUAGE: Chinese
 OTHER SOURCE: 2002-384015 [42]

AN ABB09587 Protein DGENE

AA 43 A; 43 R; 23 N; 34 D; 0 B; 12 C; 43 Q; 51 E; 0 Z; 34 G; 16 H; 26 I;
 75 L; 33 K; 25 M; 30 F; 21 P; 50 S; 30 T; 9 W; 13 Y; 50 V; 0 Others

SQL 661

SEQ

1 mkvaffgrts ngkstvinam lwdkvlpsgi ghttnclrv ggtgdgheaf1
 51 ltegseekks vktvnqlaha lhqdeqlhag slvsvmwpns kcp1lkddlv
 101 lmdspgidvt teldswidkf cldadvfvlv ansestlmqt ekqffhkvse
 ===

151 rlsrpnifil nnrwdasase peymeevrrq hmerctsflv delgvvdraq
 201 agdriffvsa kevl sarvqk aqgmpegga laegfqvrmf efqn ferrfe
 ===

251 ecisqsavkt kfeqhtvrak qiaeavrlim dslhiaaqeq rvy clemree
 301 rqdrlrfidk qlellaqdyk lrikqmt eev erqvstamae eirrlsvlvd
 ===

351 eyqmdfhpsp vvlkvyknel hrhieeglgr nmsdrcstai asslqtmqqd
 ===

401 midglkpllp vsvrnqidml vprqcfslsy dlncdklcad fgediefhfs
 451 lgwtm1vnrf lgpknrral lgyndqvgrp lpltpanpsm pplpggs1tq
 501 eelmvmvtg las1tsrtsm gilvvggvw kavgwrlial sfglyg1lyv
 551 yerltwttra kerakrqfv eyaseklqli isy1tgsncsh qvqqelsgtf
 601 ahlcqqvdit rdnleqeiaa mnkkveal1s lqskakllrn kagwldseln
 ===

651 mfihqylqps r

HITS AT: 146-148; 247-249; 343-345; 372-374; 623-625

L14 ANSWER 22 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB07031 Protein DGENE

TITLE: NEF GENE SEPARATED NOVEL HIV-2 AND PROTEIN -

INVENTOR: Kim S; Kim S; Lee H

PATENT ASSIGNEE: (GREC)GREEN CROSS CORP.

PATENT INFO: KR 155598 B1 19981015

15p

APPLICATION INFO: KR 1994-12648 19940604

PRIORITY INFO: KR 1993-10192 19930607

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 2000-314431 [27]

AN ABB07031 Protein DGENE

AA 8 A; 22 R; 4 N; 19 D; 0 B; 4 C; 12 Q; 19 E; 0 Z; 26 G; 10 H; 8 I;
20 L; 14 K; 7 M; 11 F; 19 P; 11 S; 11 T; 6 W; 10 Y; 13 V; 0 Others

SQL 254

SEQ

1 mgasgskkhs kplqglrerl lpargetcgg hcdgseegyp qsqgesgrer
51 vpsceqqrhq qgdfmntpwwk tpaterekkk yrqqnmddvd fdddddligvp
===

101 vtprvplrem tyrlavdmsh fikirgdleg mfyserrrhs rhilrkeegi
===

151 ipdwqnytyg pgvrypkffg wlwklvpvev lqlgdddgth clhlpvqtgr

201 fddlhgqtlv wrfdpmlahe ytafikypee fvyksgppeg dwkarlkarg

251 ipfn

HITS AT: 78-80; 141-143

L14 ANSWER 20 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB07033 Protein DGENE

TITLE: NEF GENE SEPARATED NOVEL HIV-2 AND PROTEIN -

INVENTOR: Kim S; Kim S; Lee H

PATENT ASSIGNEE: (GREC)GREEN CROSS CORP.

PATENT INFO: KR 155598 B1 19981015

15p

APPLICATION INFO: KR 1994-12648 19940604

PRIORITY INFO: KR 1993-10192 19930607

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 2000-314431 [27]

AN ABB07033 Protein .DGENE

AA 13 A; 21 R; 6 N; 17 D; 0 B; 3 C; 12 Q; 24 E; 0 Z; 23 G; 8 H; 9 I;
18 L; 14 K; 6 M; 10 F; 18 P; 16 S; 8 T; 7 W; 14 Y; 9 V; 0 Others

SQL 256

SEQ

1 mgasgskkhs rprrglqerl lraragacgg ywnesggeys rfqegsdreq

51 kspscegrqy qggdfmnipw kdpaaerekn lyrqgnwddv dsddddqyrv

101 svtpkvplrp mthrlaidms hliktrggle gmfyserrhk ilniylekee

=== == =

151 giiadwqnyt hpggvrypmf fgwlwklvpv dypqegedte trclyhpaqt

201 skfddphget lvwefdplla ysyefiryp eefghksglp eeekarlka

251 rgipfs

HITS AT: 113-115; 139-141

L14 ANSWER 17 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB08908 Protein DGENE

TITLE: Envelope gene of pepper mild mottle virus -

INVENTOR: Nam H; Choe J; Lee H; Park Y; Kim C

PATENT ASSIGNEE: (POHA-N)POHANG ENG COLLEGE.

PATENT INFO: KR 149216 B1 19980817 12p

APPLICATION INFO: KR 1994-29486 19941111

PRIORITY INFO: KR 1994-29486 19941111

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 2000-252824 [22]

AN ABB08908 Protein DGENE

AA 18 A; 8 R; 10 N; 7 D; 0 B; 1 C; 11 Q; 6 E; 0 Z; 8 G; 0 H; 5 I;
16 L; 2 K; 3 M; 6 F; 6 P; 11 S; 20 T; 3 W; 5 Y; 13 V; 0 Others

SQL 159

SEQ

1 maytvssanq lvylgsvwad plelqnlcts algnqfqtqq arttvqqqfs

51 dvwktiptat vrfpatgfkv lgynavldsl vsallgafdt rnriievenp

101 qnpttaetld attrvddatv airasisnlm nelvrgtgmy nqalfesasg

===

151 ltwattpyt

HITS AT: 113-115

L14 ANSWER 16 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAM48422 Protein DGENE

TITLE: Novel alkaline protease gene and expression thereof in e.coli

- NoAbstract

INVENTOR: Yu W; Lee H; Jang W; Kim E

PATENT ASSIGNEE: (KOAD)KAIST.

PATENT INFO: KR 9709083 B1 19970605

17p

APPLICATION INFO: KR 1994-9139 19940428

PRIORITY INFO: KR 1994-9139 19940428

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 1999-523621 [44]

AN AAM48422 Protein DGENE

AA 30 A; 12 R; 22 N; 11 D; 0 B; 4 C; 17 Q; 3 E; 0 Z; 35 G; 8 H; 9 I;
18 L; 2 K; 5 M; 9 F; 8 P; 34 S; 12 T; 5 W; 9 Y; 30 V; 0 Others

SQL 283

SEQ

1 aqqtpygirm vqadqlsdvy aanrkvcvid sgylrnhvdl psagvtgstf

===

51 sghgswftdg nghgthvagt ivaldnnvgv vgvlpsglv lhnvkifnds

101 gvwtrasdli qaiqscqsag shvvnmslgg sqgsvteqna mrnfyqqgml

151 lvaaagnsgn sgfsypasyd avisvaavns sgnvanfsqf nsqvelsapg

201 vnvltgng gylsysgtsm asphvagvaa lvwshfpqcr perirqslsq

251 taldrgaagr dnftgwgivq araaynwlsr ngc

HITS AT: 24-26

L14 ANSWER 23 OF 59 DGENE (C) 2002 THOMSON DERWENT
ACCESSION NUMBER: ABB07030 Protein DGENE
TITLE: MODIFIED ERYTHROPOIETIN GENE AND EXPRESSION VECTORS THEREOF

INVENTOR: Kim C; Song Y; Lee T
PATENT ASSIGNEE: (GLDS) LG CHEM CO LTD.
PATENT INFO: KR 145802 B1 19980801
APPLICATION INFO: KR 1994-12082 19940531
PRIORITY INFO: KR 1994-12082 19940531
DOCUMENT TYPE: Patent
LANGUAGE: Korean
OTHER SOURCE: 2000-234250 [20]

15p

AN ABB07030 Protein DGENE

AA 19 A; 13 R; 6 N; 6 D; 0 B; 4 C; 7 Q; 12 E; 0 Z; 9 G; 2 H; 5 I;
23 L; 8 K; 1 M; 4 F; 8 P; 10 S; 11 T; 3 W; 4 Y; 11 V; 0 Others

SQL 166

SEQ

1 apprlicdsr vlerylleak eaenittgca ehcslnenit vpdtkvnfy
51 wkrmevgqqa vevwqglall seavlrqgal lvnssqpwep lqlhvdka

===

101 glrslttllr algaqkeais ppdaasaapl rtitadtfrk lfrvysnflr

== =

151 gklklytgea crtgdr

HITS AT: 52-54; 139-141

L14 ANSWER 31 OF 59 DGENE (C) 2002 THOMSON DERWENT
ACCESSION NUMBER: ABB08497 Protein DGENE
TITLE: STABILISED RECOMBINANT ANIMAL CELL LINE EXPRESSING ENVELOPE
2

PROTEIN OF HCV
INVENTOR: Ryu W; Yang J; Cho J
PATENT ASSIGNEE: (GLDS) LG CHEM CO LTD.
PATENT INFO: KR 138597 B1 19980430
APPLICATION INFO: KR 1994-18832 19940730
PRIORITY INFO: KR 1994-18832 19940730
DOCUMENT TYPE: Patent
LANGUAGE: Korean
OTHER SOURCE: 2000-144176 [13]

8p

AN ABB08497 Protein DGENE
AA 20 A; 18 R; 17 N; 12 D; 0 B; 18 C; 9 Q; 7 E; 0 Z; 29 G; 7 H; 8 I;
28 L; 5 K; 4 M; 12 F; 25 P; 16 S; 29 T; 9 W; 13 Y; 14 V; 0 Others
SQL 300
SEQ

1 myrmqllsci alsalavtns gaqgraassl tsllfspgvpq hlqlintngs
51 whinrtalsc ndslntgfva alfykyrfna sgcperlatc rpidtfaaggw
101 gpitytephd ldqrpvcwhy apqpcgivpt lqvcgvpvcf tpspvavgtt
151 drfgaptyrw ganetdvlll nnagppqgnw fgctwmngtg ftktcggppc
201 niggvgnttl tcptdcfrkh pgatytkcgs gpwltprclv dypyrllwhyp
251 ctvnftifkv rmyvgaehr ldaacnwtrg ercdledrdr selsplllst

== =

HITS AT: 269-271

L14 ANSWER 40 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU77257 Protein DGENE

TITLE: Preparation for human adenylate kinase 2B protein - No
Abstract

INVENTOR: Choi I S; Lee Y H; Kim J H; Lee I A; Kang H B; Choi Y G; Lim
J S; Lee H G; Chung T H

PATENT ASSIGNEE: (KOAD)KOREA ADV INST SCI & TECHNOLOGY.

PATENT INFO: KR 98069616 A 19981026 18p

APPLICATION INFO: KR 1997-6762 19970228

PRIORITY INFO: KR 1997-6762 19970228

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 1999-608661 [54]

AN AAU77257 Protein DGENE

AA 21 A; 15 R; 5 N; 14 D; 0 B; 3 C; 5 Q; 18 E; 0 Z; 15 G; 5 H; 14 I;
24 L; 18 K; 8 M; 7 F; 17 P; 15 S; 12 T; 0 W; 5 Y; 11 V; 0 Others

SQL 232

SEQ

1 mapsvpaaep eypkgiravl lgppgagkgt qaprlaenfc vchlatgdml
51 ramvasgsel gkklkatmda gklvsdemvv elieknletp lckngflldg

===

101 fprrtvrqaem lddlmekrke kldsviefesi pdsllirrit grlihpksg

===

151 syheefnppk epmkdditge plirrsddne kalkirlqay htqttpliey

201 yrkrgihesai dasqtpdvvf asilaafska ts

HITS AT: 62-64; 137-139

L14 ANSWER 39 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU77262 Protein DGENE

TITLE: Novel Aquifex pyrophilus superoxide dismutase
polynucleotides

and polypeptides, used for the treatment of inflammation,
autoimmune disorders, and chromosome lesions -

INVENTOR: Han Y S; Yoo Y G; Kim S H; Lim J H; Ryoo J R; Choi I G

PATENT ASSIGNEE: (KOAD)KOREA ADV INST SCI & TECHNOLOGY.

PATENT INFO: **KR 98065913 A 19981015** 16p

APPLICATION INFO: KR 1997-1140 19970116

PRIORITY INFO: KR 1997-1140 19970116

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 1999-588588 [50]

AN AAU77262 Protein DGENE

AA 18 A; 6 R; 15 N; 12 D; 0 B; 1 C; 5 Q; 21 E; 0 Z; 15 G; 7 H; 13 I;
19 L; 20 K; 5 M; 10 F; 8 P; 5 S; 5 T; 2 W; 14 Y; 12 V; 0 Others

SQL 213

SEQ

1 mgvhklepkd hlkpqnlegi sneqiephfe ahykgyvaky neiqekladq

===

51 nfadrskanq nyseyrelkv eetfnymgvv lhelyfgmlt pggkgepsea

101 lkkkieedig gldactnelk aaamafrgwa ilgldifsgv lvnngldahn

===

151 vynltglipl ividtyehay yvdyknkrpp yidaffknin wdvvnerfek

201 amkayealkd fik

HITS AT: 4-6; 103-105

L14 ANSWER 53 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB04381 protein DGENE

TITLE: Frozen food product mfg. process - using anti-freeze peptides

ice to produce specific elongated ice crystals with desirable

recrystallisation properties and relatively hard and brittle texture

INVENTOR: Byass L J; Doucet C J; Fenn R A; McArthur A J; Sidebottom C M; Smallwood M F; Warrell D

PATENT ASSIGNEE: (UNIL) UNILEVER NV.
(UNIL) UNILEVER PLC.

PATENT INFO: WO 9822591 A2 19980528

37p

APPLICATION INFO: WO 1997-EP6181 19971106

PRIORITY INFO: EP 1996-308362 19961119

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2001-227110 [08]

AN ABB04381 protein DGENE

AA 7 A; 8 R; 15 N; 11 D; 0 B; 5 C; 7 Q; 8 E; 0 Z; 15 G; 4 H; 8 I;
33 L; 8 K; 1 M; 16 F; 16 P; 16 S; 10 T; 0 W; 3 Y; 6 V; 0 Others

SQL 197

SEQ

1 gpvplffpql tkltcldlsf nkllgvippq lstlpnlkal hlerneltge
51 ipdifgnfag spdiylshnq ltgfvpktfa radpirldfs gnrlegdisf
101 lfgpkkrlem ldfsgnvlfs nfsrvqefpp sltyldlnhn qisgslssel
===

151 akldlqtfnv sdnncgkip tggnlqrfdt taylhnsclc gaplpec

HITS AT: 106-108

L14 ANSWER 59 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB08434 protein DGENE

TITLE: Base sequence and amino acid sequence of levansucrase
derived

from Rahnella aquatilis.

INVENTOR: Lee S G; Song G B; Suh J W; Kim C H; Chung B H

PATENT ASSIGNEE: (KOAD)KOREA ADV INST SCI & TECHNOLOGY.

PATENT INFO: **KR 98077501 A 19981116** **16p**

APPLICATION INFO: KR 1997-14646 19970419

PRIORITY INFO: KR 1997-14646 19970419

DOCUMENT TYPE: Patent

LANGUAGE: Korean

OTHER SOURCE: 2000-035371 [03]

AN ABB08434 protein DGENE

AA 20 A; 18 R; 27 N; 30 D; 0 B; 4 C; 10 Q; 19 E; 0 Z; 42 G; 7 H; 21 I;
26 L; 13 K; 10 M; 18 F; 28 P; 25 S; 33 T; 9 W; 21 Y; 34 V; 0 Others

SQL 415

SEQ

1 mtnlnytpi wtradalkvn endptttqpi vdadfpvmsd evfiwdtmpl
51 rsldgtvvsv dgwsviftlt aqrnnnnsey ldaegnydit sdwnnrhgra
101 ricywysrtg kdwifggrvm aegvsptsre wagtpillne dgdidlyytc
151 vtpgatiakv rgkvltsseeg vtlagfnevk slfsadgvyy qtesqnpyn
201 frdpspfidp hdgklymvfe gnvagergsh vigkqemgtl ppghrdvna
251 ryqagcigma vakdlsqdew eilpplvtav gvndqterph fvfqdgkyy
301 ftishkftya dgltgpdgvy gflsdnltgp yspmngsglv lgnppsqpfg

===

351 tyshcvmvng lvtsfidnvp tsdgnyrigg teaptvkivl kgnrsfverv
401 fdygyippmk niiln

HITS AT: 305-307

L12 ANSWER 66 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: AAU87975 Protein DGENE

TITLE: New **endothelial** differentiation, G-protein coupled
receptor-4 gene (EDG4) polymorphic variants, for studying

the

expression and function of EDG4 and screening drugs to treat
ovarian **cancer** -

INVENTOR: Kazemi A; Koshy B; Sanchis A

PATENT ASSIGNEE: (GENA-N)GENAISSANCE PHARM INC.

PATENT INFO: WO 2002012342 A2 20020214

66p

APPLICATION INFO: WO 2001-US24649 20010806

PRIORITY INFO: US 2000-223177P 20000804

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-257470 [30]

AN AAU87975 Protein DGENE

AA 34 A; 29 R; 10 N; 7 D; 0 B; 12 C; 8 Q; 12 E; 0 Z; 20 G; 11 H; 11 I;
57 L; 4 K; 11 M; 13 F; 10 P; 27 S; 18 T; 6 W; 13 Y; 38 V; 0 Others

SQL 351

SEQ

1 mvimgqcyyn etigffynns gkelsshwrp kdvvvvalgl tvsvlvlltn
51 llviaaiasn rrfhqpiyyl lgnlaaadlf agvaylflmf htgprtarl

===

101 legwflrqgl ldtsltasva tllaiaverh rsvmavqlhs rlprgrvtml
151 ivgvwvaalg lgllpahswl clcaldrclr mapllrsyl avwalssllv
201 fllmvavytr iffyvrrrvq rmaehvschp ryrettlslv ktvviilgaf

===

251 vvcwtpgqv v lllldglgces cnvlavekyf lllaeanslv naavyscrda
301 emrrtfrll ccacrlrqstr esvhytssaq ggastrimlp enghplmdst

===

351 1

HITS AT: 61-63; 217-219; 307-309

L12 ANSWER 2 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB79643 Protein DGENE

TITLE: Treating, delaying the onset of, or ameliorating an inflammatory pathology (e.g. inflammatory bowel disease or Crohn's disease) by administering to the subject fibroblast growth factors or a combination of growth factors -

INVENTOR: Jeffers M; Shimkets R A; Prayaga S; Boldog F L; Yang M; Burgess C E; Fernandes E R; Rittman B; Shimkets J B; Larochelle W J; Lichenstein H S

PATENT ASSIGNEE: (CURA-N)CURAGEN CORP.

PATENT INFO: WO 2002058716 A2 20020801 196p

APPLICATION INFO: WO 2001-US43846 20011106

PRIORITY INFO: US 2000-246206P 20001106

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-599742 [64]

AN ABB79643 Protein DGENE

AA 17 A; 33 R; 22 N; 26 D; 0 B; 15 C; 12 Q; 23 E; 0 Z; 15 G; 9 H; 20 I; 29 L; 20 K; 3 M; 15 F; 17 P; 30 S; 22 T; 5 W; 16 Y; 21 V; 0 Others

SQL 370

SEQ

1 mhrlifvytl icanfcscrd tsatpqsasi kalrnanlrr desnhltdly

===

51 rrdeti qvkg ngyvqsprfp nsyprnlllt wrlhsqentr iqlvfdnqfg

101 leeaendicr ydfvevedis etstiirgrw cghkevppri ksrtngikit

151 fksddyfvak pgfkiiysll edfqpaaase tnwesvtssi sgvsynspsv

201 tdptliadal dkkiaefdtv edllkyfnpe swqedlenmy ldtprrygrs

===

251 yhdrkskvd1 drlnddakry sctprnysvn ireelklanv vffprcllvq

===

301 rcggnccgct vnwrstcns gktvkkyhev lqfepghikr rgraktmalv

===

351 diqldhherc dcicssrppr

HITS AT: 2-4; 212-214; 268-270; 325-327

L12 ANSWER 13 OF 69 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB80921 Protein DGENE

TITLE: Identifying agents for modulating (e.g. inhibiting) e.g. **tumor** establishment, growth or metastases, neuron growth, or wound healing by determining whether a test compound binds with a 22437 polypeptide (human sulfatase) -

INVENTOR: Glucksmann M A; Rudolph-Owen L A

PATENT ASSIGNEE: (MILL-N)MILLENNIUM PHARM INC.

PATENT INFO: WO 2002052019 A2 20020704 143p

APPLICATION INFO: WO 2001-US30856 20011003

PRIORITY INFO: US 2000-257082P 20001221

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 2002-566677 [60]

AN ABB80921 Protein DGENE

AA 36 A; 59 R; 48 N; 52 D; 0 B; 24 C; 38 Q; 40 E; 0 Z; 59 G; 35 H; 30 I;
85 L; 68 K; 24 M; 31 F; 43 P; 54 S; 48 T; 15 W; 40 Y; 41 V; 0 Others

SQL 870

SEQ

```
1 mgppslvlcl lsatvfsllg gssafllshhr lkgrfqrdrn nirpniilvl
      == =
51 tddqdvelgs mqvmnkttrri meqggthfin afvttpmccp srssiltgky
      ===
101 vhnhtytnn encsspswqa qhesrtfavy lntgyrtaf fgkylneyng
151 syvppgwkew vgllknsrfy nytlcrngvk ekhgsdyskd yltldlitnds
201 vsffrtskkm yphrpvlmvi shaaphged sapqysrlfp nasqhitpsy
      ===
251 nyapnpdkhw imrytgpmkp ihmeftnmlq rkrlqtlmsv ddsmetiynm
      ===
301 lvetgeldnt yivytadhgy higqfglvkg ksmpeyefdir vpfyvrgpnv
351 eagclnphiv lnidlaptil diagldipad mdgksilkll dterpvnrfh
401 lkkkmrvwrđ sflvergkll hkrđndkvda qeenflpkyq rvkdldcrae
      ===
451 yqtaceqlgq kwqcvedatg klklhkckgp mrlggsrals nlvpkygggq
501 seactcdsgđ yklslagrrk klfkkykas yvrsrsirsv aievdgrvyh
      = == ===
551 vglgdaaqpr nltkrhwpga pedqddkdgg dfsgtgglpd ysāanpikvt
      ===
601 hrcyilendť vqcdldlyks lqawkdhklh idheietlqn kiknlrevrg
      ===
651 hlkkkrpeec dchkisyhtq hkgrlkhrgs slhpfrkglq ekdkvllre
      ===
701 qkrkkklrkl lkrlqnndtc smpgltcfth dnqhwqtapf wtlgpfcať
      =====
751 sannntywcm rtinethnfl fcefatgfle yfdlntdpyq lmnvntldr
801 dvlnqlhvql melrsckgyk qcnprtrnmd lgldggsye qyrqfqrkw
      ===
851 pemkrpssks lgqlwegweg
```

HITS AT: 29-31; 68-70; 208-210; 258-260; 282-284; 403-405; 520-522; 525-527;
565-567; 627-629; 663-665; 705-707; 708-710; 712-714; 848-850

L25 ANSWER 19 OF 27 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB79049 peptide DGENE

TITLE: Diagnosing cancer and apoptotic disease from distribution of
Rad51 foci in cells - also detecting cells with mutant Rad51
genes, screening for agents that bind Rad51 and inducing
apoptosis by increasing Rad51 activity

INVENTOR: Haff T; Radding T; Reddy C; Guruward D

PATENT ASSIGNEE: (UYYA)UNIV YALE.

PATENT INFO: **WO 9834118 A1 19980806**

73p

APPLICATION INFO: WO 1998-US1825 19980130

PRIORITY INFO: US 1997-35834P 19970130

US 1997-45668P 19970506

US 1998-7020 19980114

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 1998-437609 [37].

AN ABB79049 peptide DGENE

AA 0 A; 2 R; 0 N; 0 D; 0 B; 0 C; 0 Q; 3 E; 0 Z; 0 G; 0 H; 0 I;

0

L; 2 K; 0 M; 0 F; 0 P; 0 S; 1 T; 0 W; 1 Y; 0 V; 0 Others

SQL 9

SEQ

1 eekrkrtye

===

HITS AT: 5-7

L25 ANSWER 20 OF 27 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB79047 peptide DGENE

TITLE: Diagnosing cancer and apoptotic disease from distribution of Rad51 foci in cells - also detecting cells with mutant Rad51 genes, screening for agents that bind Rad51 and inducing apoptosis by increasing Rad51 activity

INVENTOR: Haff T; Radding T; Reddy C; Guruward D

PATENT ASSIGNEE: (UYYA)UNIV YALE.

PATENT INFO: WO 9834118 A1 19980806

73p

APPLICATION INFO: WO 1998-US1825 19980130

PRIORITY INFO: US 1997-35834P 19970130

US 1997-45668P 19970506

US 1998-7020 19980114

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 1998-437609 [37]

AN ABB79047 peptide DGENE

AA 1 A; 4 R; 0 N; 0 D; 0 B; 0 C; 0 Q; 0 E; 0 Z; 0 G; 0 H; 0 I;
0

L; 0 K; 0 M; 0 F; 1 P; 0 S; 0 T; 0 W; 0 Y; 0 V; 0 Others

SQL 6

SEQ

1 arrrrp

===

HITS AT: 4-6

L14 ANSWER 11 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB79046 peptide DGENE

TITLE: Diagnosing cancer and apoptotic disease from distribution of Rad51 foci in cells - also detecting cells with mutant Rad51 genes, screening for agents that bind Rad51 and inducing apoptosis by increasing Rad51 activity

INVENTOR: Haff T; Radding T; Reddy C; Guruward D

PATENT ASSIGNEE: (UYYA)UNIV YALE.

PATENT INFO: WO 9834118 A1 19980806

73p

APPLICATION INFO: WO 1998-US1825 19980130

PRIORITY INFO: US 1997-35834P 19970130

US 1997-45668P 19970506

US 1998-7020 19980114

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 1998-437609 [37]

AN ABB79046 peptide DGENE

AA 0 A; 1 R; 0 N; 0 D; 0 B; 0 C; 0 Q; 0 E; 0 Z; 0 G; 0 H; 0 I;
0

L; 4 K; 0 M; 0 F; 1 P; 0 S; 0 T; 0 W; 0 Y; 1 V; 0 Others

SQL 7

SEQ

1 pkkkrrkv

===

HITS AT: 5-7

proteoglycan

L14 ANSWER 10 OF 59 DGENE (C) 2002 THOMSON DERWENT

ACCESSION NUMBER: ABB79050 peptide DGENE

TITLE: Diagnosing cancer and apoptotic disease from distribution of Rad51 foci in cells - also detecting cells with mutant Rad51 genes, screening for agents that bind Rad51 and inducing apoptosis by increasing Rad51 activity

INVENTOR: Haff T; Radding T; Reddy C; Guruward D

PATENT ASSIGNEE: (UYYA)UNIV YALE.

PATENT INFO: WO 9834118 A1 19980806

73p

APPLICATION INFO: WO 1998-US1825 19980130

PRIORITY INFO: US 1997-35834P 19970130

US 1997-45668P 19970506

US 1998-7020 19980114

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: 1998-437609 [37]

AN ABB79050 peptide DGENE

AA 5 A; 1 R; 0 N; 1 D; 0 B; 0 C; 1 Q; 0 E; 0 Z; 1 G; 0 H; 0 I;

1

L; 7 K; 0 M; 0 F; 1 P; 0 S; 1 T; 0 W; 0 Y; 1 V; 0 Others

SQL 20

SEQ

1 avkrpaatk agqakkkld

===

HITS AT: 17-19

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(54) Title: DIAGNOSTIC METHODS AND COMPOSITIONS BASED ON THE DISTRIBUTION OF RAD51 (57) Abstract Method of diagnosing individual at risk for a disease comprising determining the distribution of RAD51 foci in a tissue type of a first individual; and comparing said distribution of RAD51 foci form a second normal tissue type from said first individual or a second unaffected individual.		

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DIAGNOSTIC METHODS AND COMPOSITIONS BASED ON THE DISTRIBUTION OF RAD51

This is a continuing application of 60/035,834, filed January 30, 1997 and 60/045,668, filed May 6, 1997, both of which are expressly incorporated by reference herein.

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FIELD OF THE INVENTION

The invention relates to methods of diagnosis and screening utilizing Rad51 molecules.

BACKGROUND OF THE INVENTION

Homologous recombination is a fundamental process which is important for creating genetic diversity and for maintaining genome integrity. In *E.coli* RecA protein plays a central role in homologous genetic recombination *in vivo* and promotes homologous pairing of double-stranded DNA with single-stranded DNA or partially single-stranded DNA molecules *in vitro*. Radding, C. M. (1988). Homologous pairing and strand exchange promoted by Escherichia coli RecA protein. Genetic Recombination. Washington, American Society for Microbiology. 193-230; Radding, C. M. (1991). J. Biol. Chem. 266: 5355-5358; Kowalczykowski, et al., (1994). Annu. Rev. Biochem. 63: 991-1043. In the yeast *Saccharomyces cerevisiae* there are several genes with homology to recA gene; Rad51, Rad57 and Dmc1. Rad51 is a member of the Rad52 epistasis group, which includes Rad50, Rad51, Rad52, Rad54, Rad55 and Rad57. These genes were initially identified as being defective in the repair of damaged DNA caused by ionizing radiation and were subsequently shown to be deficient in both genetic recombination and the recombinational repair of DNA lesions. Game, J. C. (1983). Yeast Genetics: Fundamental

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and applied aspects. J.F.T. Spencer, D.H. Spencer and A.R.W. Smith, eds (New-York:springer-verlag) : 109-137; Haynes, et al., (1981). The molecular biology of the yeast *Saccharomyces cerevisiae*: Life cycle and inheritance. J.N. Strathern, E.W. Jones and J.M. Broach, eds (Cold Spring harbor, New York:Cold Spring Harbor laboratory press) : 371-414; Resnick, M. A. (1987). Meiosis, P.B. Moens, ed. (New York: Academic Press) : 157-210. During meiosis Rad51 mutants accumulate DNA double-strand breaks at recombination hot spots (Shinohara, et al., (1992). Cell 69: 457-470). Yeast rad51 gene was cloned and sequenced (Basile, et al., (1992). Mol. Cell. Biol. 12: 3235-3246; Aboussekhar, et al., (1992) Mol. Cell. Biol. 12: 3224-3234). Although yeast Rad51 gene shared homology with *E.coli* recA gene, the extent of homology was not very strong (27%). However, the extent of structural conservation between RecA protein and Rad51 protein became apparent when the yeast Rad51 protein was isolated and was shown to form nucleoprotein filaments that were almost identical to the nucleoprotein filaments formed by RecA protein (Ogawa, et al., (1993). CSH Symp. Quant. Biol. 58: 567-576; Ogawa, T., et al., (1993). Science 259: 1896-1899; Story, et al., (1993). Science 259: 1892-1896). Recently genes homologous to *E.coli* recA and yeast rad51 were isolated from all groups of eukaryotes, including mammals (Morita, et al., (1993). Proc. Natl. Acad. Sci. USA 90, 6577-6580; Shinohara, et al., (1993). Nature Genet. 4, 239-243; Heyer, W.D. (1994). Experientia 50, 223-233; Maeshima, et al., (1995). Gene 160: 195-200). Phylogenetic analysis by Ogawa and co workers suggested the existence of two sub-families within eukaryotic RecA homologs: the Rad51-like (Rad51 of human, mouse, chicken, *S. cerevisiae*, *S. pombe* and Mei3 of *Neurospora crassa*) and the Dmc1-like genes (*S. cerevisiae* Dmc1 and *Lilium longiflorum* LIM15) (Ogawa, supra). All these Rad51 genes share significant homology with residues 33-240 of the *E.coli* RecA protein, which have been identified as a 'homologous core' region.

Yeast and human Rad51 proteins have been purified and characterized biochemically. Like *E.coli* RecA protein, yeast and human Rad51 protein polymerizes on single-stranded DNA to form a right-handed helical nucleoprotein filament which extends DNA by 1.5 times (Story, supra; Benson, et al., (1994) EMBO J. 13, 5764-5771). Moreover like RecA protein Rad51 protein promotes homologous pairing and strand exchange in an ATP dependent reaction (Sung, P. (1994). Science 265, 1241-1243; Sung, P. and D. L.

Robberson (1995). Cell 82: 453-461; Baumann, et al., (1996) Cell 87, 57-766; Gupta, et al., (1997) Proc. Natl. Acad. Sci. USA 94, 463-468). Surprisingly, polarity of strand exchange performed by Rad51 protein is opposite to that of RecA protein (Sung and Robberson supra) and the relevance of this observation remains to be seen.

- 5 Surprisingly, studies with mouse models show that targeted disruption of the Rad51 gene leads to an embryonic lethal phenotype (Tsuzuki, et al., (1996). Proc. Natl. Acad. Sci. USA 93: 6236-6240). Moreover attempts to generate homozygous rad51^{-/-} embryonic stem cells have not been successful. These results show that Rad51 plays an essential role in cell proliferation, a surprise in view of the viability of *S.cerevisiae* carrying rad51
- 10 deletions. It is also interesting to note that Rad51 was found to be associated with RNA polymerase II transcription complex (Maldonado, et al., (1996). Nature 381, 86-89), the specificity and functional nature of these interactions remains to be seen but all these observations point to a pleiotropic role of hsRad51 in DNA metabolism.

- While Rad51 transcripts and protein are present in all the cell types examined thus
- 15 far, the highest transcript levels are found in tissues active in recombination, including spleen, thymus, ovary and testis (Morita, supra). Rad51 is specifically induced in murine B cells cultured with lipopolysaccharide, which stimulates switch recombination and Rad51 localizes to nuclei of switching B cells (Li, et al., (1996). Proc. Natl. Acad. Sci. USA 93: 10222-10227). These findings are consistent with the
- 20 view that Rad51 plays an important role in lymphoid specific recombination events such as V(D)J recombination and immunoglobulin heavy chain class switching. In spermatocytes undergoing meiosis, Rad51 is enriched in the synaptonemal complexes, which join paired homologous chromosomes (Haaf, et al., (1995) Proc. Natl. Acad. Sci. USA 92, 2298-2302; Ashley, et al., (1995) Chromosoma 104:
- 25 19-28; Plug, et al., (1996). Proc. Natl. Acad. Sci. USA 93: 5920-5924). In cultured human cells, Rad51 protein is detected in multiple discrete foci in the nucleoplasm of a few cells by immunofluorescent antibodies. After DNA damage, the localization of Rad51 changes dramatically when multiple foci form in the nucleus and stain vividly with anti-Rad51 antibodies (Haaf, supra, 1995). After DNA

damage the percentage of cells with focally concentrated Rad51 protein increases; the same cells show unscheduled DNA-repair synthesis.

Micronuclei (MN) originate from chromosomal material that is not incorporated into daughter nuclei during cell division. Different chemicals and treatment of cells induce qualitatively different types of micronuclei. MN caused by ionizing radiation or clastogens (i.e. 5-azacytidine) mostly contain acentric chromosome fragments (Verhaegen, F., and Vral, A. (1994). *Radiation Res.* 139, 208-213; Stopper, et al., (1995). *Carcinogenesis* 16, 1647-1650). In contrast, MN induced by aneuploidogens (i.e. colcemid) result from lagging whole chromosomes and stain positively for the presence of kinetochores/ centromeres (Marrazini et al., 1994; Stopper, et al., (1994). *Mutagenesis* 9, 411-416). Determination of MN frequencies represents a good assay to measure genetic damage in cells, since it is much faster and simpler than karyotype analyses. In this light, the MN test has been widely used as a dosimeter of human exposure to radiation or clastogenic and aneugenic chemicals, and for the detection and risk assessment of environmental mutagens and carcinogens (Heddle, et al., (1991) *Environmental Mol. Mutagenesis* 18, 277-291; Norppa, et al., (1993). *Environmental Health Perspect.* 101, Supp. 3, 139-143; Hahnfeldt, et al., (1994) *Radiation Res.* 138, 239-245). However, although the MN assay is a convenient in situ method to monitor cytogenetic effects, the understanding of the connection between initial DNA damage and formation of MN is still poor.

The tumor suppressor p53 prevents tumor formation after DNA damage by halting cell cycle progression to allow DNA repair or by inducing apoptotic cell death. Loss of wild-type p53 function renders cells resistant to DNA damage induced cell cycle arrest and ultimately leads to genomic instabilities including gene amplifications, translocations and aneuploidy. Some of these chromosomal lesions are based on mechanisms that involve recombinational events (Lane, D. P. (1992). *Nature* 358: 15-16; Lane, D. P. (1993). *Nature* 362: 786-787; Sturzbecher, et al., (1996). *EMBO J.* 15: 1992-2002) reported that wild-type tumor suppressor protein p53 interacts physically with human Rad51 protein and it inhibits the biochemical functions of

- Rad51 like ATPase and strand exchange. In vivo temperature sensitive mutant p53 formed complexes with Rad51 only in wild type but not in mutant conformation. They suggested that gene amplifications and other types of chromosome rearrangements involved in tumour progression might occur not only as a result of inappropriate cell proliferation but as a direct consequence of a defect in p53 mediated control of homologous recombination processes due to mutations in the p53 gene. (Meyn, et al., (1994). Int. J. Radiat. Biol. 66: S141-S149) showed that normal cells transfected with a dominant-negative p53 mutant acquired interference with the G1-S cell cycle checkpoint and showed up to an 80-fold elevation in RAD51 mediated homologous DNA recombination rates compared with the normal parental control cells. Thus, loss of normal p53 function may cause a loss in control of normal DNA repair, recombination, and ultimately replication, resulting in inappropriate cell division and neoplastic growth. Breast tumour cells have mutated p53 genes and proteins and have various types of chromosomal aberrations like insertions, deletions, rearrangements, amplifications etc., indicative of abnormally controlled recombination.

Accordingly, it is an object of the invention to provides methods of diagnosis and screening which focus on Rad51.

SUMMARY OF THE INVENTION

- In accordance with the objects outlined above, the present invention provides methods of diagnosing individuals at risk for a disease state which results in aberrant Rad51 loci. The methods comprise determining the distribution of Rad51 foci in a first tissue type of a first individual, and then comparing the distribution to the distribution of Rad51 foci from a second normal tissue type from the first individual or a second unaffected individual. A difference in the distributions indicates that the first individual is at risk for a disease state which results in aberrant Rad51 loci. Preferred disease states include cancer and disease states associated with apoptosis.

In an additional aspect, the present invention provides methods for identifying apoptotic cells and cells under stress associated with nucleic acid modification. The methods comprise determining the distribution of Rad51 foci in a first cell, and comparing the distribution to the distribution of Rad51 foci from a second non-
5 apoptotic cell. A difference in the distributions indicates that the first cell is apoptotic or under stress.

In a further aspect, the present invention provides methods for identifying a cell containing a mutant Rad51 gene comprising determining the sequence of all or part of at least one of the endogenous Rad51 genes.

10 In an additional aspect, the invention provides methods of identifying the Rad51 genotype of an individual comprising determining all or part of the sequence of at least one Rad51 gene of the individual. The method may include comparing the sequence of the Rad51 gene to a known Rad51 gene.

In a further aspect, the present invention provides methods for screening for a
15 bioactive agent capable of binding to Rad51. The methods comprise adding a candidate bioactive agent to a sample of Rad51, and determining the binding of the candidate agent to the Rad51.

In an additional aspect, the invention provides methods for screening for a bioactive agent capable of modulating the activity of Rad51. The method comprises the steps
20 of adding a candidate bioactive agent to a sample of Rad51, and determining an alteration in the biological activity of Rad51. The method may also comprise adding a candidate bioactive agent to a cell, and determining the effect on the formation or distribution of Rad51 foci in the cell.

In a further aspect, the invention provides methods of inducing apoptosis in a cell
25 comprising increasing the activity of Rad51 in the cell. This can be done by overexpressing an endogenous Rad51 gene, or by administration of a gene encoding Rad51 or the protein itself.

In an additional aspect, the present invention provides composition comprising a nucleic acid encoding a Rad51 protein, and a nucleic acid encoding a tumor suppressor protein. The tumor suppressor protein may be p53 or a BRCA protein.

BRIEF DESCRIPTION OF THE DRAWINGS

- 5 Figure 1 is a digital image of photographs of cells that depict type I and type II Rad51 foci, respectively.

Figures 2A and 2B are digital images of photographs of two different breast cancer cells from a breast cancer cell line (BT20) that show Rad51 foci. The staining is localized to the nucleus, and does not occur in either the cytoplasm or the nucleolus.

- 10 Figures 3A, 3B, 3C and 3D show dynamic changes in the higher-order nuclear organization of Rad51 foci after DNA damage and cell-cycle arrest. (a-c) TGR-1 fibroblasts were irradiated with a lethal dose (900 rad) of ^{137}Cs and then allowed to recover for various times. Rad51 protein is stained (light), nuclei are counterstained with DAPI. Three hours after irradiation (a), Rad51 foci are distributed throughout
15 the entire nuclear volume. Many foci have a double-dot appearance. After 16 hrs (b), clusters of Rad51 foci and linear higher-order structures are formed. Somatic pairing of linear strings of Rad51 foci is observed. After 30 hrs (c), Rad51 clusters move towards the nuclear periphery and are eliminated into micronuclei. (d) Simultaneous staining of Rad51 protein (light) and replicating DNA (dark) in an
20 exponentially growing, XPA fibroblast culture. BrdU was incorporated into DNA for 30 hrs and detected with red anti-BrdU antibody. Note that the Rad51-positive cell is devoid of BrdU label. Magnification 1000x.

- Figure 4 depicts the exclusion of Rad51 -protein in micronuclei after DNA damage. TGR-1 fibroblasts, two days after ^{137}Cs irradiation with a dose of 900 rad. Rad51
25 protein is stained by (light), nuclei are counterstained with DAPI. Note the complete absence of Rad51-protein staining in nuclei. All Rad51 foci are excluded into

micronuclei. Most micronuclei exhibit paired Rad51-protein structures.

Magnification 1000x.

Figures 5A, 5B, 5C and 5D illustrates that apoptotic bodies (micronuclei) contain Rad51 protein and fragmented DNA. (a and b) TGR-1 nuclei, 3 hrs (right), 16 hrs (middle), and 30 hrs (left) after ^{137}Cs irradiation. Rad51-protein foci show light staining. The repair proteins Rad52 (a) and Gadd45 (b) are detected by antibody probes (darker staining). Nuclei are counterstained with DAPI. Note that neither Rad52 nor Gadd45 foci co-localize with Rad51. Only the Rad51 foci segregate into micronuclei. (c and d) Micronuclei induced by treatment of TGR-1 cultures with colcemid (c) and etoposide (d) contain Rad51 protein (light staining, left nucleus) and fragmented DNA (darker staining, right nucleus). Magnification 1000x.

Figures 6A, 6B and 6C show the association of Rad51 protein with linear DNA molecules. (a) Mechanically stretched chromatin prepared from a ^{137}Cs -irradiated cell culture and stained with light anti-HsRad51 antibodies. The Rad51 signals appear as beads-on-a-string on the linearly extended chromatin fibers. (b and c) DNA fibers excluded from TGR-1 nuclei, one day after ^{137}Cs irradiation. Preparations are not experimentally stretched. Chromatin is counterstained with DAPI. The DNA fibers are covered with Rad51 protein (c, light staining), whereas the remaining nuclei are devoid of detectable Rad51 foci. DNA-strand breaks in chromatin fibers are end labeled with fluorescent nucleotides (c, darker staining co-localizing with the Rad51 staining). Some fibers appear to form micronuclei. Magnification 1000x.

Figures 7A, 7B, 7C, 7D, 7E and 7F show the linear higher-order structures of Rad51 protein in overexpressing nuclei and in colcemid-induced micronuclei. Rad51 protein is stained with anti-Rad51 antiserum, detected by green FITC fluorescence (light staining). Preparations are counterstained with DAPI, except the nucleus in b. (a and b) Human 710 kidney cells overexpressing Rad51 fused to a T1-tag epitope. Nuclei are filled with a network of linear Rad51 structures. Magnification 1000x. (c) Subconfluent rat TGR 928.1-9 cells overexpressing HsRad51. Nuclear staining is

most prominent in cells during G₀ and G₁ phase of the cell cycle. Magnification 1000x. (d) TGR 928.1-9 nucleus filled with linear Rad51 structures. Magnification 1000x. (e and f) Linear Rad51 structures in colcemid-induced micronuclei. TGR-1 fibroblasts were treated with colcemid for one day and then allowed to recover for
5 two days. Note the absence of Rad51 staining in the nuclei. Magnification 1000x.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a series of discoveries relating to the pivotal role that Rad51 plays in a number of cellular functions, including those involved in disease states. Thus, it appears that the levels, function, and distribution of the
10 Rad51 protein within cells may be monitored as a diagnostic tool of cellular health or fate. In addition, due to Rad51's essential role in a number of cellular processes, Rad51 is an important target molecule to screen candidate drug agents which can modulate its biological activity.

Accordingly, in a preferred embodiment, the invention provides methods of
15 diagnosing individuals at risk for a disease state. As will be appreciated by those in the art, "at risk for a disease state" means either that an individual has the disease, or is at risk to develop the disease in the future. By "disease state" herein is meant a disease that is either caused by or results in aberrant Rad51 distribution or biological activity. For example, as is more fully described below, aberrant distribution of
20 Rad51 foci in a cell can be indicative of cancer, apoptosis, cellular stress, etc., which can lead to the development of disease states. Similarly, disease states caused by or resulting in aberrant Rad51 biological activity, including alterations caused by mutation, changes in the cellular amount or distribution of Rad51, and changes in the biological function of Rad51, for example altered nucleic acid binding, filament
25 formation, DNA pairing (i.e. D-loop formation), strand-exchange, strand annealing or recombination, are also included within the definition of disease states which are related to or associated with Rad51.

Thus, disease states which may be evaluated using the methods of the present invention include, but are not limited to, cancer (including solid tumors such as skin, breast, brain, cervical carcinomas, testicular carcinomas, etc.), diseases associated with premature or incorrect apoptosis, including AIDS, cancers (e.g. melanoma, hepatoma, colon cancer, etc.), liver failure, Wilson disease, myelodysplastic syndromes, neurodegenerative diseases, multiple sclerosis, aplastic anemia, chronic neutropenia, Type I diabetes mellitus, Hashimoto thyroiditis, ulcerative colitis, Canale-Smith syndrome, lymphoma, leukemia, solid tumors, and autoimmune diseases), diseases associated with cellular stress which is affiliated with nucleic acid modification, including diseases associated with oxidative stress such as cardiovascular disease, immune system function decline, aging, brain dysfunction and cancer.

In one embodiment, the method comprises first determining the distribution of Rad51 foci in a first tissue type of a first individual, i.e. the sample tissue for which a diagnosis is required. In some embodiments, the testing may be done on single cells. The first individual, or patient, is suspected of being at risk for the disease state, and is generally a human subject, although as will be appreciated by those in the art, the patient may be animal as well, for example in the development or evaluation of animal models of human disease. Thus other animals, including mammals such as rodents (including mice, rats, hamsters and guinea pigs), cats, dogs, rabbits, farm animals including cows, horses, goats, sheep, pigs, etc., and primates (including monkeys, chimpanzees, orangutans and gorillas) are included within the definition of patient.

As will be appreciated by those in the art, the tissue type tested will depend on the disease state under consideration. Thus for example, potentially cancerous tissue may be tested, including breast tissue, skin cells, solid tumors, brain tissue, etc. Similarly, cells or tissues of the immune system, including blood, and lymphocytes; cells or tissues of the cardiovascular system (for example, for testing oxidative stress).

In a preferred embodiment, the disease state under consideration is cancer and the tissue sample is a potentially cancerous tissue type. Of particular interest is breast, skin, brain, colon, prostate, and other solid tumor cancers. As outlined in the Examples, cultured breast cancer cells and primary invasive breast cancer cells all
5 demonstrate an increase in the presence of Rad51 foci.

Similarly, several diseases caused by defective nucleotide excision repair (NER) systems, including Xeroderma pigmentosum, show increased Rad51 foci.

In a preferred embodiment, primary cancerous tissue is used, and may show differential Rad51 staining. While the number of cells exhibiting Rad51 foci may be
10 less than for cell lines, primary cancerous tissue shows an increase in Rad51 foci. Thus for example, from 0.05 to 10% of primary cancerous cells exhibit differential Rad51 foci, with from about 1 to about 5% being common.

It should be noted that not all cancer cell lines exhibit aberrant Rad51 protein foci. For example, the ovarian cancer cell line Hey does not show an increase in Rad51
15 foci. Similarly, as outlined in the examples, transformed but non-malignant human cells can show an increased percentage of Rad51-positive cells (compared to non-transformed cells), although it is generally not as great as in tumor cells.

In a preferred embodiment, the disease state under consideration involves apoptosis, and includes, but is not limited to, including AIDS, cancers (e.g. melanoma,
20 hepatoma, colon cancer, etc.), liver failure, Wilson disease, myelodysplastic syndromes, neurodegenerative diseases, multiple sclerosis, aplastic anemia, chronic neutropenia, Type I diabetes mellitus, Hashimoto thyroiditis, ulcerative colitis, Canale-Smith syndrome, lymphoma, leukemia, solid tumors, and autoimmune diseases. This list includes disease states that include too much as well as too little
25 apoptosis. See Peter et al., PNAS USA 94:12736 (1997), hereby incorporated by reference.

In a preferred embodiment, the disease state under consideration involves cellular stress associated with nucleic acid modification, including aging, cardiovascular disease, declines in the function of the immune system, brain dysfunction, and cancer.

- 5 The distribution of Rad51 foci is determined in the target cells or tissue. To date, two main types of Rad51 foci have been identified. As reported earlier (Haaf, 1995, supra) *in situ* immunostaining with Rad51 antibodies reveals three kinds of nuclei: 1) nuclei that did not show any staining at all (no foci); 2) nuclei that showed weak to medium staining and showed only a few foci (Type I nuclei); and 3) nuclei that
- 10 showed strong staining and showed many foci (Type II nuclei). In general, the staining is excluded from the cytoplasm. Type I and Type II patterns of nuclei staining are shown in Figure 1; many of the foci have a double-dot appearance, typical of paired DNA segments. In normal cells, type I nuclei are found in 7-10% of cells and type II nuclei in less than 0.4 to 1% of cells, with generally about 90%
- 15 of the cells showing no foci. In contrast, some cells involved in disease states show a marked increase in Rad51 foci. As outlined herein and shown in the examples, the numbers of cells showing Rad51 foci in cells associated with disease states is significantly increased. Thus, in a preferred embodiment, the number of cells showing type I nuclei is generally from about 5% to about 50% of the nuclei, with
- 20 from about 10% to about 40% generally being seen. Thus, in a preferred embodiment, there is at least a 5% increase in the type I foci, with at least about 10 % being preferred, and at least about 30% being particularly preferred. Generally, to see this effect, at least about 100 cells should be evaluated, with at least about 500 cells being preferred, and at least about 1000 being particularly preferred.
- 25 Similarly, the number of cells showing type II nuclei also increases, with from about 1% to about 10% of the nucleic exhibiting type II foci and from about 1% to about 5% being common. Thus, in a preferred embodiment, there is at least a 5% increase in type II foci, with at least about 10% being preferred, and at least about 30% being particularly preferred. In a preferred embodiment, both types of foci increase
- 30 simultaneously. In alternate embodiments, only one type of foci increases.

Similarly, an increase in both types of foci (i.e. an increase in any foci, irrespective of type) can also be evaluated using the same numbers.

- The distribution of Rad51 foci can be determined in a variety of ways. In a preferred embodiment, a labeled binding agent that binds to Rad51 is used to visualize the foci. By "labeled" herein is meant that a compound has at least one element, isotope or chemical compound attached to enable the detection of the compound. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) immune labels, which may be antibodies or antigens; and c) colored or fluorescent dyes. The labels may be incorporated into the compound at any position. Preferred labels are fluorescent or radioactive labels. The binding agent can either be labeled directly, or indirectly, through the use of a labeled secondary agent which will bind to the first binding agent. The cells or tissue sample is prepared as is known for cellular or *in situ* staining, using techniques well known in the art, as outlined in the Examples.
- 15 In a preferred embodiment, the binding agent used to detect Rad51 foci is an antibody. The antibodies may be either polyclonal or monoclonal, with monoclonal antibodies being preferred. In general, it is preferred, but not required, that antibodies to the particular Rad51 under evaluation be used; that is, antibodies directed against human Rad51 are used in the evaluation of human patients.
- 20 However, as the homology between different mammalian Rad51 molecules is quite high (73% identity as between human and chicken, for example), it is possible to use antibodies against Rad51 from one type of animal to evaluate a different animal (mouse antibodies to evaluate human tissue, etc.). Thus, in a preferred embodiment, antibodies raised against eukaryotic Rad51 are used, with antibodies raised against mammalian Rad51 being especially preferred. Thus, antibodies raised against yeast,
- 25 human, rodent, primate, and avian Rad51 proteins are particularly preferred. In addition, as will be appreciated by those in the art, the protein used to generate the antibodies need not be the full-length protein; fragments and derivatives may be used, as long as there is sufficient immunoreactivity against the sample Rad51 to

allow detection. Alternatively, other binding agents which will bind to Rad51 at sufficient affinity to allow visualization can be used.

Without being bound by theory, as outlined in the Examples, it does not appear that the quantitative amount of Rad51 protein is necessarily altered in cells exhibiting the presence or altered distribution of foci. However, in some circumstances the quantitative amount of Rad51 may be measured and correlated to the presence or absence of Rad51 foci.

In addition, the appearance of the foci may be used in the determination of the presence of aberrant Rad51 foci. As noted in the Examples, in some cases linear “strings” of 5-10 Rad51 foci are formed, with somatic association of “homologous” strings of similar length, tightly paired at one of the ends. These structures are generally associated with DNA fibers, as is shown in the Figures. Thus, the formation of these types of structures can be indicative of aberrant Rad51 foci.

Furthermore, in a preferred embodiment, particularly in disease states involving apoptosis and DNA damage, aberrant Rad51 foci includes the development of micronuclei containing Rad51. As shown in the Examples, evaluation of Rad51 foci over time, in particular after cellular stress, can lead to the concentration and exclusion of the Rad51 foci (which are associated with DNA) into micronuclei, which frequently is accompanied by genome fragmentation. This effect is seen in a wide variety of apoptotic cells, as is shown in the Examples, even in the absence of induced DNA damage, such as through the use of colcemid, a spindle poison, thus indicating the role of Rad51 in normal apoptotic pathways.

In addition to the evaluation of the presence or absence of Rad51 foci, the cells may be evaluated for cell cycle arrest, as is outlined in the Examples.

Once the distribution of Rad51 foci has been determined for the target sample, the distribution of foci is compared to the distribution of Rad51 foci from a second cell or tissue type. As will be appreciated by those in the art, the second tissue sample can be from a normal cell or tissue from the original patient or a tissue from another,

unaffected individual, which has been matched for correlation purposes. A difference in the distribution of Rad51 foci as between the first tissue sample and the second matched sample indicates that the first individual is at risk for a disease state which results in aberrant Rad51 loci.

- 5 In a preferred embodiment, the difference in Rad51 foci distribution is an increase in Rad51 foci, of either type 1 or type 2 foci, as outlined above. In an alternate embodiment, the difference in Rad51 foci distribution is a decrease in the number of Rad51 foci.

- 10 In some embodiments, there need not be a direct comparison. For example, having once shown that a particular normal tissue only contains a small percentage of Rad51 foci, the tissue or cells under evaluation may not need to be compared to a control sample; the presence of a higher percentage allows the diagnosis. Thus, for example, in breast cancer, the presence of at least 1% of the cells containing Rad51 foci is indicative that the patient is at risk for breast cancer or in fact already has it.

- 15 In a preferred embodiment, a difference in the distribution of Rad51 foci, in particular an increase in Rad51 foci, indicates that the cell or tissue is cancerous.

- In a preferred embodiment, a difference in the distribution of Rad51 foci, in particular an increase in Rad51 foci, indicates that the cell or tissue is apoptotic. These differences can include the association of Rad51 with DNA fibers, the
20 association of Rad51 with damaged DNA in micronuclei, or the presence of Rad51 in micronuclei.

In addition, in a preferred embodiment, the extent of aberrant distribution indicates the severity of the disease state. Thus, for example, high percentages of cells containing Rad51 foci can be indicative of highly malignant cancer.

- 25 In addition to the evaluation of Rad51 foci, the presence or absence of variant (mutant) Rad51 genes may also be used in diagnosis of disease states. Mutant forms

of p53 have been found in roughly 50% of known cancers, and it is known that Rad51 and p53 can interact on a protein level. In addition, p53 and Rad51 have somewhat similar biochemical functions. Thus, the present discovery that Rad51 plays a pivotal role in some cancers and apoptosis thus suggests that variant Rad51, or incorrectly controlled Rad51 levels or functions may be important in some disease states.

Accordingly, in a preferred embodiment, the present invention provides methods for identifying a cell containing a mutant Rad51 gene comprising determining the sequence of all or part of at least one of the endogenous Rad51 genes. By “variant Rad51 gene” herein is meant any number of mutations which could result in aberrant Rad51 function or levels. Thus, for example, mutations which alter the biochemical function of the Rad51 protein, alter its half-life and thus its steady-state cellular level, or alter its regulatory sequences to cause an alteration in its steady-state cellular level may all be detected. This is generally done using techniques well known in the art, including, but not limited to, standard sequencing techniques including sequencing by PCR, sequencing-by-hybridization, etc.

Similarly, in a preferred embodiment, the present invention provides methods of identifying the Rad51 genotype of an individual or patient comprising determining all or part of the sequence of at least one Rad51 gene of the individual. This is generally done in at least one tissue of the individual, and may include the evaluation of a number of tissues or different samples of the same tissue. For example, putatively cancerous tissue of an individual is the preferred sample.

The sequence of all or part of the Rad51 gene can then be compared to the sequence of a known Rad51 gene to determine if any differences exist. This can be done using any number of known homology programs, such as Bestfit, etc.

In a preferred embodiment, the presence of a difference in the sequence between the Rad51 gene of the patient and the known Rad51 gene is indicative of a disease state or a propensity for a disease state.

The present discovery relating to the role of Rad51 in cancer and apoptosis thus provide methods for inducing apoptosis in cells. In a preferred embodiment, the methods comprise increasing the activity of Rad51 in the cells. By "biological activity" of Rad51 herein is meant one of the biological activities of Rad51, including, but not limited to, the known Rad51 DNA dependent ATPase activity, the nucleic acid strand exchange activity, the formation of foci, single-stranded and double-stranded binding activities, filament formation (similar to the recA filament of yeast), pairing activity (D-loop formation), etc. See Gupta et al., supra, and Bauman et al., supra, both of which are expressly incorporated by reference herein.

As will be appreciated by those in the art, this may be accomplished in any number of ways. In a preferred embodiment, the activity of Rad51 is increased by increasing the amount of Rad51 in the cell, for example by overexpressing the endogenous Rad51 or by administering a gene encoding Rad51, using known gene-therapy techniques, for example. In a preferred embodiment, the gene therapy techniques include the incorporation of the exogenous gene using enhanced homologous recombination (EHR), for example as described in PCT/US93/03868, hereby incorporated by reference in its entirety.

In a preferred embodiment, the cells which are to have apoptosis induced are cancer cells, including, but not limited to, breast, skin, brain, colon, prostate, testicular, ovarian, etc. cancer cells, and other solid tumor cells.

In a preferred embodiment, the methods may also comprise subjecting the cells to conditions which induce nucleic acid damage, as this appears to provide a synergistic effect, as outlined above.

In a preferred embodiment, the methods further comprise increasing the activity of p53 in the cell, for example by increasing the amount of p53, as outlined above for Rad51.

The present discoveries relating to the pivotal role of Rad51 in a number of important cellular processes and disease states also makes Rad51 an important target

in drug screening. Thus, in a preferred embodiment, the present invention provides methods for screening for a bioactive agent which may bind to Rad51 and modulate its activity.

In a preferred embodiment, the methods are used to screen candidate bioactive agents for the ability to bind to Rad51. In this embodiment, the methods comprise adding a candidate bioactive agent to a sample of Rad51 and determining the binding of the candidate agent to the Rad51. By "candidate bioactive agent" or "candidate drugs" or grammatical equivalents herein is meant any molecule, e.g. proteins (which herein includes proteins, polypeptides, and peptides), small organic or inorganic molecules, polysaccharides, polynucleotides, etc., which are to be tested for the capacity to bind and/or modulate the activity of Rad51. Candidate agents encompass numerous chemical classes. In a preferred embodiment, the candidate agents are organic molecules, particularly small organic molecules, comprising functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more chemical functional groups.

Candidate agents are obtained from a wide variety of sources, as will be appreciated by those in the art, including libraries of synthetic or natural compounds. Any number of techniques are available for the random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications to produce structural analogs.

In a preferred embodiment, candidate bioactive agents include proteins, nucleic acids, and organic moieties.

- In a preferred embodiment, the candidate bioactive agents are proteins. By "protein" herein is meant at least two covalently attached amino acids, which includes
- 5 proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the
- 10 invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard in vivo degradations.
- 15 In a preferred embodiment, the candidate bioactive agents are naturally occurring proteins or fragments of naturally occurring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eukaryotic proteins may be made for screening against Rad51. Particularly preferred in this embodiment
- 20 are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

- In a preferred embodiment, the candidate bioactive agents are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides
- 25 may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any

nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized candidate bioactive proteinaceous agents.

- 5 In one embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for
- 10 example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking. prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

- In a preferred embodiment, the candidate bioactive agents are nucleic acids. By
- 15 "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate (Beaucage et al., Tetrahedron 49(10):1925
- 20 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent
- 25 No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphosphoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al.,
- 30 Nature 380:207 (1996), all of which are incorporated by reference). Other analog

nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine, etc.

As described above generally for proteins, nucleic acid candidate bioactive agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of procaryotic or eucaryotic genomes may be used as is outlined above for proteins.

In a preferred embodiment, the candidate bioactive agents are organic chemical moieties, a wide variety of which are available in the literature.

The candidate agents are added to a sample of Rad51 protein. As is outlined above, all or part of a full-length Rad51 protein can be used, or derivatives thereof.

- 5 Generally, the addition is done under conditions which will allow the binding of candidate agents to the Rad51 protein, with physiological conditions being preferred.

The binding of the candidate agent to the Rad51 sample is determined. As will be appreciated by those in the art, this may be done using any number of techniques.

- 10 In one embodiment, the candidate bioactive agent is labelled, and binding determined directly.

- Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescent molecules, enzyme reporters, colorimetric reporters, chemiluminescers, specific binding molecules, particles, e.g. magnetic or gold particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxigenin and antidigoxigenin etc. For the specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures.

- 20 In some embodiments, only one of the components is labeled. For example, the Rad51 may be labeled at tyrosine positions using ^{125}I . Alternatively, more than one component may be labeled with different labels; using ^{125}I for the Rad51, for example, and a fluorophor for the candidate agents.

- 25 In a preferred embodiment, the binding of the candidate bioactive agent is determined directly. For example, the Rad51 may be attached to a solid support such as a microtiter plate or other solid support surfaces, and labelled candidate

agents added under conditions which favor binding of candidate agents to the Rad51 protein. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high through put screening.

- 5 Typically between 0.1 and 1 hour will be sufficient. Excess reagents are washed off, the system is evaluated for the presence of the label, which is indicative of an agent which will bind to the Rad51. The agent which binds can then be characterized or identified as needed.

- In a preferred embodiment, the binding of the candidate bioactive agent is
10 determined through the use of competitive binding assays. In this embodiment, the competitor is can be any molecule known to bind to Rad51, for example an antibody to Rad51, or one of the proteins known to interact with Rad51, including Rad52, Rad54, Rad55, DMC 1, BRCA1, BRCA2, p53, UBC9, RNA polymerase II, and Rad51 itself, any or all of which may be used in competitive assays. Either the
15 candidate agents or the competitor may be labeled, or both may be labeled with different labels. In this embodiment, either the candidate bioactive agent, or the competitor, is added first to the Rad51 sample for a time sufficient to allow binding, if present, as outlined above. Excess reagent is generally removed or washed away. The second component is then added, and the presence or absence of the labeled
20 component is followed, to indicate binding.

- In a preferred embodiment, methods for screening for a bioactive agent capable of modulating the activity of Rad51 comprise the steps of adding a candidate bioactive agent to a sample of Rad51, as above, and determining an alteration in the biological activity of Rad51. "Modulating the activity of Rad51" includes an increase in
25 activity, a decrease in activity, or a change in the type or kind of activity present. Thus, in this embodiment, the candidate agent should both bind to Rad51 (although this may not be necessary), and alter its biological or biochemical activity as defined above.

Thus, in this embodiment, the methods comprise combining a Rad51 sample and a candidate bioactive agent, and testing the Rad51 biological activity as is known in the art to evaluate the effect of the agent on the activity of Rad51.

In a preferred embodiment, the methods include both in vitro screening methods, as
5 are generally outlined above, and in vivo screening of cells for alterations in the presence, distribution or activity of Rad51. Accordingly, in a preferred embodiment, the methods comprise the steps of adding a candidate bioactive agent to a cell, and determining the effect on the formation or distribution of Rad51 foci in the cell. The addition of the candidate agent to a cell will be done as is known in the art, and may
10 include the use of nuclear localization signal (NLS). NLSs are generally short, positively charged (basic) domains that serve to direct the entire protein in which they occur to the cell's nucleus. Numerous NLS amino acid sequences have been reported including single basic NLS's such as that of the SV40 (monkey virus) large T Antigen (Pro Lys Lys Lys Arg Lys Val), Kalderon (1984), et al., Cell, 39:499-509;
15 the human retinoic acid receptor- β nuclear localization signal (ARRRRP); NF κ B p50 (EEVQRKRQKL; Ghosh et al., Cell 62:1019 (1990); NF κ B p65 (EEKRKRTYE; Nolan et al., Cell 64:961 (1991); and others (see for example Bouliskas, J. Cell. Biochem. 55(1):32-58 (1994), hereby incorporated by reference) and double basic NLS's exemplified by that of the Xenopus (African clawed toad)
20 protein, nucleoplasmin (Ala Val Lys Arg Pro Ala Ala Thr Lys Lys Ala Gly Gln Ala Lys Lys Lys Lys Leu Asp), Dingwall, et al., Cell, 30:449-458, 1982 and Dingwall, et al., J. Cell Biol., 107:641-849; 1988). Numerous localization studies have demonstrated that NLSs incorporated in synthetic peptides or grafted onto reporter
25 proteins not normally targeted to the cell nucleus cause these peptides and reporter proteins to be concentrated in the nucleus. See, for example, Dingwall, and Laskey, Ann, Rev. Cell Biol., 2:367-390, 1986; Bonnerot, et al., Proc. Natl. Acad. Sci. USA, 84:6795-6799, 1987; Galileo, et al., Proc. Natl. Acad. Sci. USA, 87:458-462, 1990. In general, the Rad51 foci will be evaluated as is generally discussed above.

In a preferred embodiment, the methods comprise adding a candidate bioactive agent
30 to a cell, and determining the effect on double strand break repair, homologous

recombination, sensitivity to ionizing radiation, and class switch recombination.

Assays are detailed in Park, J. Biol. Chem. 270(26):15467 (1995) and Li et al., PNAS USA 93:10222 (1996), Shinohara et al., supra, 1992, all of which are hereby incorporated by reference.

- 5 In a preferred embodiment, the cells to which candidate agents are added are subjected to conditions which induce nucleic acid damage, including the addition of radioisotopes (I^{125} , Tc, etc., including ionizing radiation and uv), chemicals (Fe-EDTA, bis(1,10-phenanthroline), etc.), enzymes (nucleases, etc.).

a variety of other reagents may be included in the screening assays or kits, below.

- 10 These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. In general, the mixture of components may
15 be added in any order that provides for the requisite binding.

- Once identified, the compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host, as previously described. The inhibitory agents may be administered in a variety of ways, orally, parenterally e.g., subcutaneously, intraperitoneally, intravascularly, etc. Depending
20 upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.

- The pharmaceutical compositions can be prepared in various forms, such as granules, aerosols, tablets, pills, suppositories, capsules, suspensions, salves, lotions
25 and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and

emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

In a preferred embodiment, kits are provided. The kits can be utilized in a variety of applications, including determining the distribution of Rad51 foci, diagnosing an individual at risk for a disease state, including cancer, diseases associated with apoptosis, and diseases associated with stress (including oxidative stress, hypoxic stress, osmotic stress or shock, heat or cold stress or shock). The kits include a Rad51 binding agent, that will bind to the Rad51 with sufficient affinity for assay. Antibodies are preferred binding agents. The kits further include a detectable label such as is outlined above. In one embodiment, the Rad51 binding agent is labeled; in an additional embodiment, a secondary binding agent or label is used. Thus for example, the binding agent may include biotin, and the secondary agent can include streptavidin and a fluorescent label. Additional reagents such as outlined above can also be included. Furthermore, the kit may include packaging and instructions, as required.

The identification of the crucial role of Rad51 in a number of cellular processes and disease states also identifies a number of methods and compositions relating to combinations of Rad51 and other tumor suppressor genes. Thus, Rad51 may function interactively with a number of tumor suppressor genes and thus compositions comprising combinations of these genes may be useful in methods of gene therapy treatment and diagnosis.

Accordingly, in a preferred embodiment, compositions comprising a nucleic acid encoding a Rad51 protein and at least one nucleic acid encoding a tumor suppressor gene are provided. Suitable tumor suppressor genes include, but are not limited to, p53, and the BRCA genes, including BRCA1 and BRCA2 genes. Thus, preferred embodiments include compositions of nucleic acids encoding a) a Rad51 gene and a p53 gene; b) a Rad51 gene and a BRCA1 gene; c) a Rad51 gene and a BRCA2 gene; d) a Rad51 gene, a p53 gene, and a BRCA gene; and e), a Rad51 gene, a p53 gene, a BRCA1 gene and a BRCA2 gene.

In an additional embodiment, the compositions comprise recombinant proteins. By “recombinant” herein is meant a protein made using recombinant techniques, i.e. through the expression of a recombinant nucleic acid as depicted above. a recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host, and thus may be substantially pure. For example, an isolated protein is unaccompanied by at least some of the material with which it is normally associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of the total protein in a given sample. a substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 80% being preferred, and at least about 90% being particularly preferred. The definition includes the production of a protein from one organism in a different organism or host cell. Alternatively, the protein may be made at a significantly higher concentration than is normally seen, through the use of a inducible promoter or high expression promoter, such that the protein is made at increased concentration levels. Alternatively, the protein may be in a form not normally found in nature, as in the addition of an epitope tag or amino acid substitutions, insertions and deletions, as discussed below.

20 In a preferred embodiment, these compositions can be administered to a cell or patient, as is outlined above and generally known in the art for gene therapy applications.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are specifically incorporated by reference.

EXAMPLES

Example 1

Immunofluorescent Staining of Human Breast Cancer Cells

Breast tumour cells have mutated p53 and have various types of chromosomal

- 5 aberrations like insertions, deletions, rearrangements, amplifications etc.

Recombination proteins such as Rad51 could evidently participate in such processes.

In order to better understand the role of uncontrolled recombination and its role in tumour formation and progression, the status of Rad51 protein in breast tumour cells by staining them with anti Rad51 antibodies was done.

- 10 Detailed methods of cloning and expression of HsRad51 gene in *E.coli*, purification of recombinant HsRad51 protein with six histidine residues at its aminoterminal end and preparation of polyclonal antibodies against HsRad51 protein were described previously by Haaf, Golub et al. 1995, supra, which is expressly incorporated herein by reference.
- 15 **Immunofluorescent staining with anti-Rad51 protein antibodies.** Monolayer cultures of different cell substrates (see table 1) were grown in Dulbecco's MEM medium supplemented with 10% fetal bovine serum and antibiotics. The cells were detached from culture flasks by gentle trypsinization, pelleted and resuspended in phosphate buffered saline (PBS; 136 mM NaCl, 2 mM KCl, 10.6 mM Na₂HPO₄, 1.5
- 20 mM KH₂PO₄ [pH 7.3]) prewarmed at 37°C. For immunofluorescence staining standard protocols were used (Haaf 1995, supra). Cultured cells were washed and resuspended in PBS. The density of somatic cells was adjusted to about 10⁵ cells per ml in PBS. Aliquots (0.5 ml) of the cell suspension were centrifuged onto clean glass slides at 800 rpm for 4 min, in a Cytospin (Shandon, Pittsburg). Immediately after
- 25 cytocentrifugation, the slides were fixed in -20°C methanol for 30 min and then immersed in ice-cold acetone for a few seconds to permeabilize the cells for antibody staining. Following three washes with PBS, the preparations were incubated at 37°C with rabbit anti-HsRad51 antiserum, diluted 1:50 with PBS containing 0.5% bovine serum albumin, in a humidified incubator for 30 min. The slides were washed three

times for 10 min each and then incubated for 30 min with fluorescein-isothiocyanate (FITC)- conjugated anti-rabbit IgG diluted 1:20 with PBS. After three washes with PBS, the preparations were counterstained with 4',6-diamidino-2- phenylindole (DAPI; 0.1 ug/ml for 1 min) and mounted in antifade {90% (vol/vol) glycerol/0.1 m tris-HCl pH 8.0/2.3% 1,4-diazabicyclo[2.2.2]octane (DABCO)}.

Digital Imaging Microscopy. Images were taken with a Zeiss epifluorescence microscope with a thermo-electronically cooled charge coupled device (CCD) camera (model PM512; Photometrics, Tucson, AZ) which was controlled by an Apple Macintosh computer. Grey scale source images were captured separately with filter sets for fluorescein and DAPI. Gray scale source images were pseudocolored and merged using ONCOR Image and ADOBE Photoshop software. It is worth emphasizing that although a CCD imaging system was used, all antibody signals were clearly visible by eye through the microscope.

To study the possible involvement of Rad51 in tumorigenesis we compared the in situ localization of Rad51 protein homologs in different cell substrates i.e. mortal fibroblast strains, virus-transformed non-malignant cell lines and tumor cell lines (see table). a specific rabbit antiserum raised against human Rad51 protein was used in these studies. These antibodies reacted mainly with Rad51 protein in mammalian cell extracts as judged by Western blotting (see fig No 2 in (Haaf, Golub et al. 1995). Immunostaining of different cells showed that HsRad51 is concentrated in small and discrete sites (foci) through out nucleoplasm and is largely excluded from nucleoli and cytoplasm. At least 250 nuclei of exponentially growing cultures were analyzed for each experiment. As reported earlier (Haaf, Golub et al. 1995) immunostaining revealed three kinds of nuclei: 1) nuclei that did not show any staining at all (no foci), 2) nuclei that showed weak to medium staining and showed only a few foci (Type I nuclei) 3) nuclei that showed strong staining and showed many foci (Type II nuclei). In normal fibroblast control cells, we found type I nuclei in about 10% of cells and type II nuclei in less than 0.4 to 1% of cells and about 90% of the cells showed no foci. Use of preimmune serum, as well as omission of

either the primary or secondary antibody, resulted in the absence of focally concentrated nuclear immunofluorescence.

As reported earlier (Haaf, Golub et al. 1995) in normal (mortal) fibroblast control cells (Hs68) we found type I nuclei in 7% -10% of cells and type II nuclei in less than 0.4% of cells, where as 90% or more of the cells showed no foci (Table 1). In contrast all breast tumor cell lines tested (BT20, SrBr3, MoF7) exhibited 1-5% of type II nuclei and 10-38% of type I nuclei (Table 1). Transformed but non-malignant human cells, i.e. SV 40 transformed fibroblasts (LNL8, 63L7), EBV-transformed lymphoblasts (GM 01194), and adenovirus-transformed kidney cells (293) also showed an increased percentage of Rad51-positive cells (compared to normal fibroblasts), however the numbers observed were lower than in tumor cells. Interestingly, some tumor substrates i.e. the ovarian cancer line Hey; did not show a significant increase of Rad51-positive cells.

As demonstrated earlier (Haaf, Golub et al. 1995), when the normal fibroblast cells were exposed to DNA damaging agents like 137Cs, there was a significant increase of cells containing type I and type II nuclei (Table 2). It is worth emphasizing that non-irradiated breast tumor cells show approximately the same percentage of Rad51-positive nuclei as Hs68 fibroblasts exposed to 900 rad Cs137 which kills 99% of cells (Table 2). The immunofluorescent patterns of (non-irradiated) breast cancer cells (Figure 1) and fibroblasts that were exposed to DNA damaging agents are identical.

When the breast cancer cells were exposed to Cs137, the increase in the number of cells with type I and type II nuclei was even more dramatic than in normal (Hs68) or transformed (LNL8) fibroblasts (Table 2). Up to 40% of irradiated breast cancer cells showed type I nuclei and 11%-18% showed type II nuclei.

In order to rule out any artifacts that would arise due to the examination of cultured breast cancer cells, we then examined the breast tissue obtained directly from the patient for Rad51 positive staining. Immunohistochemical evaluation revealed

- definite nuclear staining of invasive breast carcinoma cells. Specifically, nuclear reactivity could be demonstrated in sections obtained from three paraffin-embedded samples. The nuclear staining appeared granular in some areas, and in others, occupied the entire nucleus. The actual number of invasive carcinoma cells that
- 5 fluoresced was quite small, and estimated to be less than 5% of the nuclei seen in three samples with definite reactivity Figure 2). Nuclear staining was not identical in normal breast epithelium or lactating breast tissue. Bright nuclear reactivity was seen in positive control testicular tissue, specifically, in the cells lining the seminiferous tubules. Background staining did not appear to be problematic.
- 10 Increase in immunofluorescence of HsRad51 in breast cancer cells can result from either increase in the amount of Hsrad51 in these cells or it could be seen as a result of re-organization of Hsrad51 in these nuclei in response to damage related activities. We think that the latter is true because there was no apparent increase in the amount HsRad51 in breast cancer cells as shown by the Western blots (data not
- 15 shown).

- The molecular basis and the consequence of the increase in HsRad51 in breast cancer cells is not clear. Since Rad51 protein interacts with other proteins of the Rad52 epistasis group and these multiprotein complexes are involved in the recombinational repair of double-strand breaks (Hays, et al., (1995). Proc. Natl.
- 20 Acad. Sci. USA 92: 6925-6929; Johnson, R. D. and L. S. Symington (1995). Mol. Cell. Biol. 15: 4843-4850), it is tempting to speculate that these foci are the sites where repair/recombination events are taking place. Since p53 is known to interact with Rad51 it will be interesting to see the colocalization of p53 and Rad51 protein in these complexes. It is quite possible that these foci contain either wild type or
- 25 mutant p53 and other breast cancer related proteins like BRCA1, BRCA2 or the newly discovered STG1 protein. We propose that the increase in the immunofluorescence of Rad51 in the breast cells can be used as an important cytological marker for cell proliferation and malignant cell growth. Further experimentation will be done to validate this proposal and to understand the role of
- 30 increase in Rad51 foci and carcinogenesis.

Table 1: Percentage of nuclei containing discrete foci enriched with HsRad51 protein.

Cell Substrate			No foci	Type I	Type II
5	Hs68	Normal fibroblasts	90%	10%	0%
			93%	7%	0%
	LNL8 (NI 00847)	Transformed fibroblasts	90%	9%	1%
		(SV 40)	90%	8%	2%
10	63L7	Transformed fibroblasts	94%	6%	0%
		(SV40)	94%	3%	3%
	GM01194	Transformed lymphoblasts	91%	7%	2%
		(EBV)	90%	9%	1%
			92%	8%	0%
			80%	18%	2%
15			80%	19%	1%

WO 98/34118			PCT/US98/01825	
293 Cells	Transformed kidney cells	75%	23%	2%
	(Adenovirus)	83%	15%	2%
		82%	17%	1%
5 BT20	Breast cancer line	86%	10%	4%
		82%	13%	5%
		78%	17%	5%
SrBr3	Breast cancer line	74%	25%	1%
MoF7	Breast cancer line	57%	38%	5%
		88%	10%	2%
10 Tera2	Testicular teratoma	76%	23%	1%
		77%	22%	1%
Hey	Ovarian cancer line	94%	5%	1%
		98%	2%	0%
HeLa	Cervix (?) tumor cells	67%	31%	2%

Table 2: Percentage of nuclei containing discrete foci enriched with HsRad51 protein.

	Cell substrate	Treatment	No foci	Type I	Type II
5	Hs68	None	90%	10%	0%
	(normal	None	93%	7%	0%
	fibroblasts)	6 hrs after 10 rad Cs137	96%	4%	0%
		6 hrs after 50 rad Cs137	96%	4%	0%
		6 hrs after 150 rad Cs137	92%	7%	1%
10		6 hrs after 450 rad Cs137	88%	8%	4%
		6 hrs after 900 rad Cs 137	91%	4%	5%
	LNL8(NI 00847)	None	90%	9%	1%
	(SV40-transformed	None	90%	8%	2%
	fibroblasts)	6 hrs after 150 rad Cs137	88%	11%	1%
		6 hrs after 300 rad Cs137	76%	19%	5%
		6 hrs after 900 rad Cs137	78%	17%	5%
15	BT20	None	86%	10%	4%
	(breast cancer	None	82%	13%	5%
	cells)	None	78%	17%	5%
20		6 hrs after 300 rad Cs137	44%	41%	11%
		6 hrs after 900 rad Cs137	52%	30%	18%

Example 2**Nuclear foci of human recombination protein Rad51****in nucleotide excision repair defective cells**

- Eukaryotic cells have several different mechanisms for repairing damaged DNA
- 5 (for review see R. Wood, 1996). One of the major pathway is nucleotide excision repair (NER), which excises damage within oligomers that are 25-32 nucleotides long. Patients with recessive heredity disorder XP have defects in one of several enzymes, which participate in ER. There are seven XP groups (XP-A to XP-G), which have defects in the initial steps of the DNA excision repair.
- 10 DNA damage is removed several-fold faster from transcribed genes than from non-transcribed, mainly due to preferential NER of the transcribed strand (for review see Hanawalt, 1994). This mechanism does not function in Cockayne's syndrome (CS) patients.

NER defective cells, evidently, sustain increased amount of DNA damage. Thus we evaluated NER defective cells from XP and CS cells for an increased amount of Rad51 protein foci.

To study possible effect of NER on localization of HsRad51 in somatic tissue culture cells, we compare in situ localization of the protein in normal fibroblasts, different XP cells and CS-B cells. A polyclonal rabbit antiserum raised against human Rad51 protein was used in this study. These antibody reacted in mammalian cell extract mainly with Rad51 protein as judged by Western Blotting (see FIG. 2 in Haaf et al., 1995). Immunostaining of different cell lines showed that HsRad51 is concentrated in small and discrete sites (foci) throughout nucleoplasm and is largely excluded from nucleoli and cytoplasm. As discussed above, immunostaining revealed three kinds of nuclei, types I, II and III. The results are shown in Table 3.

Table 3: Percentage of nuclei containing discrete foci enriched with HsRad51 protein

		Cell substrate	No foci	Type I*	Type II*
5	Hs68	Normal fibroblasts	90%	10%	0%
		Normal fibroblasts			
	63L7		94%	6%	0%
	63L7 (confluent)	FA fibroblasts	94%	3%	3%
	6935	FA fibroblasts	92%	6%	2%
10	6914	Normal lymphoblasts	72%	21%	7%
	6914		72%	25%	3%
	6914		67%	24%	9%
	GM01194	Normal lymphoblasts	91%	7%	2%
	GM01194		90%	9%	1%
15	GM01194		92%	8%	0%
		FA lymphoblasts			
	GM07063		90%	8%	2%
	GM07063	FA lymphoblasts	96%	4%	0%
	GM13020	FA lymphoblasts	92%	7%	1%
20	GM13022		86%	13%	1%
	GM13022		78%	20%	2%
	GM13023		94%	5%	1%
	GM13071		81%	15%	4%
	GM13071		74%	23%	3%

*Type I nuclei show only few (<15) foci and/or weak to medium HsRad51 immunofluorescence, whereas Type II cells show many and/or strongly fluorescing foci.

250 nuclei were analyzed for each experiment.

In normal (mortal) fibroblast control cells, LNL8 and NF, we found type I nuclei in 5-9% cells and type II nuclei in 1-7% cells, where as 88-90% of the cells showed no foci (Table 3). Use of preimmune serum, as well as omission of either the primary or secondary antibody, resulted in the absence of focally concentrated nuclear immunofluorescence.

XP-V cells are normal in NER, but have defect in postreplication repair process (Boyer et al., 1990; Griffiths et al., 1991; Wang et al., 1991, 1993). As we expected, these cells showed the same distribution pattern of nuclear HsRad51 as control cell lines (Table 3).

Distribution of HsRad51 foci in CS-B cells also was similar to the cells with normal NER (Table 3). This result was also anticipated. CS-B cells are defective in NER which is coupled with transcription (Venema et al., 1990). Transcribed genes, evidently, comprise only a small part of the whole genomic DNA and damage in transcribed genes, therefore, should be accounted for only a very small fraction of the damage in genomic DNA.

XP-A, XP-B, XP-F and XP-G cells are all defective in NER. XP-A cells have defect in XPA protein, which carries out a crucial rate-limiting step in NER-recognition of DNA lesion (Jones and Wood, 1993). The protein makes a ternary complex with ERCC1 protein and XPF protein, which is defective in XP-F cells (Park and Sankar, 1994). XP-B and XP-G cells are defective in different steps of NER which follow damage recognition (Reviewed in Ma et al., 1995).

XP-A and XP-F cell lines have increased amount of cells with HsRad51 protein foci (Table 3). In contrast, XP-B and XP-G cells have about the same level of HsRad51 protein foci, as cells with normal NER (Table 3). This result could be easily understood if we assume, that 1) formation of HsRad51 foci is caused by DNA damage, b) DNA lesion is excluded from the pool of damage DNA which cause Rad51 foci formation as soon as XPA/XPF/ERCC1 complex binds to the lesion. DNA damage in XP-B and XP-G cells is recognized by NER system, but the damage

cannot be proceeded and removed by the system. Such unremoved damage, evidently, is not considered as a substrate for Rad51 protein involved repair as soon as the damage is recognized by NER complex XPA/XPF/ERCC1 as a substrate for NER, even if defect in subsequent steps of NER makes its removing impossible.

- 5 Induction of principal DNA repair system (SOS respond) in *E. coli* is, assumed to be triggered by formation of single-stranded DNA (ssDNA) which results from DNA damage (reviewed in Little and Mount, 1982). DNA damage in XP-A cells is not recognized by NER and, therefore, at least a considerable part of DNA damage is not proceeded to formation of ssDNA regions. Nevertheless, Rad51 foci are effectively
- 10 formed in XP-A cells and their amount could be further increased by UV or - irradiation (Tables 4 and 5). Evidently, ssDNA is not a primary signal for HsRad51 protein foci formation.

Table 4: Percentage of nuclei containing discrete foci enriched with HsRad51 protein

	Cell substrate	Treatment	No foci	Type I*	Type II*
5	LNL8 (control)	No treatment	90%	9%	1%
		"	90%	8%	2%
	NF (control)	No treatment	88%	5%	7%
		"	89%	5%	6%
	XPA	"	51%	39%	10%
10		No treatment	72%	20%	8%
		"	55%	34%	7%
	XPB	No treatment	86%	11%	3%
		"	86%	11%	3%
	XPD	No treatment	87%	8%	5%
		"	63%	28%	9%
	XPF	No treatment	48%	41%	11%
		"	64%	25%	8%
	XPG	None	88%	7%	5%
			85%	9%	6%
	XPV		94%	5%	1%
		89%	11%	0%	
	CBS		87%	8%	5%

*Type I nuclei show only a few (<15) foci and/or weak to medium HsRad51 immunofluorescence, whereas type II cells show many and/or strongly fluorescing foci.

250 nuclei were analyzed for each experiment.

Table 5: Percentage of nuclei containing discrete foci enriched with HsRad51 protein

Cell substrate	Treatment	No foci	Type I*	Type II*
5 LNL8 (control)	No treatment	90%	9%	1%
	No treatment	90%	8%	2%
	6 hrs after 150 rad Cs137	88%	11%	1%
	6 hrs after 300 rad Cs137	76%	19%	5%
	6 hrs after 900 rad Cs137	78%	17%	5%
	None	51%	39%	10%
	3 hrs after 300 rad ¹³⁷ Cs	61%	24%	15%
	None	72%	20%	8%
	6 hrs after 900 rad ¹³⁷ Cs	59%	25%	16%
	None	59%	34%	7%
	5 hrs after 5 J/m ² UV	53%	31%	16%
	5 hrs after 15 J/m ² UV	55%	26%	19%
	None	87%	8%	5%
	5 hrs after 800 rad ¹³⁷ Cs	60%	21%	19%
	27 hrs after 800 rad ¹³⁷ Cs	77%	6%	17%
XPA**				
CBS**				

*Type I nuclei show only a few (<15) foci and/or weak to medium HsRad51 immunofluorescence, whereas Type II cells show many and/or strongly fluorescing foci.

150 nuclei were analyzed for each experiment.

5 **Induction of HsRad51 foci in Xeroderma pigmentosum (Type A) implies that single stranded DNA molecules are not the primary signal.

***Induction of HsRad51 foci in cells from patients with Cockayne's syndrome implies that the induction is not dependent on transcription.

In conclusion, human recombination protein HsRad51 is concentrated in multiple
10 discrete foci in nucleoplasm of cultured human cells. After treatment of cells with DNA damaging agents, the percentage of cells with HsRad51 protein immunofluorescence increases. Xeroderma pigmentosum (XP) cells XP-A with inactive protein XPA, responsible for lesion recognition by nucleotide excision repair (NER) system have increased percentage of cells with HsRad51 protein foci.
15 XP-F cells, defective in XPF protein, which forms complex with XPA protein, also have increased level of the HsRad51 protein foci. In contrast, XP-B and XP-G cells with defects in different steps ER, which follow the damage recognition, as well as XP-V cells (normal level of NER) and Cockayne's syndrome (CS) cells (defect in NER, responsible for preferential repair of the transcribed DNA strand) have normal
20 level of HsRad51 protein foci. Evidently, formation of HsRad51 protein foci is caused by DNA damages. DNA damages, however, do not participate in causing formation of HsRad51 protein foci, as soon as they are recognized by NER system, even if the system is blocked on one of the step, leading to DNA repair.

Example 3

25 Higher order nuclear structures of Rad51 and its exclusion into micronuclei after cell damage

Previous studies have revealed a time- and dose-dependent increase of nuclear HsRad51 protein foci after DNA damage introduced into the genome by various agents (Haaf et al., 1995, supra). Here we show that when the damaged cells are
30 allowed to recover, these Rad51 foci form specific higher-order nuclear structures. Finally, all the focally concentrated Rad51 protein is eliminated into micronuclei that undergo apoptotic genome fragmentation. Treatment of cells with clastogens

and aneuploidogens implements a mechanism that affects the nuclear distribution of Rad51 protein and targets Rad51 foci, most likely along with irreversibly damaged DNA into micronuclei. To examine the role of Rad51 protein in DNA repair and cell proliferation, we have analyzed the intranuclear distribution of overexpressed

5 Rad51 protein during the cell cycle and in cell populations proceeding through apoptosis.

Experimental Procedures

Cell Culture. The sources of the cell lines were as follows. Rat TGR-1 cells, J. Sedivy, Brown University; mouse 3T3-Swiss cells, ATCC; human 293 kidney cells,

10 ATCC; human teratoma cells, B. King, Yale University; human LNL8 fibroblasts, S. Meyn, Yale; human XPA and XPF fibroblasts, P Glazer, Yale.

Monolayer cultures were grown in D-MEM medium supplemented with 10% fetal bovine serum and antibiotics. The cells were detached from culture flasks by gentle trypsinization, pelleted and resuspended in phosphate-buffered saline (PBS; 136 mM

15 NaCl, 2 mM KCl, 10.6 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3) prewarmed at 37°C.

To induce DSBs in DNA and recombinational repair, cell cultures were exposed to a ¹³⁷Cs irradiator at doses of 900 rad and then allowed to recover for various time spans. In another experiment, cells were treated with 10 μM 5-aza-dC for 24 hrs.

20 This hypomethylating base analog is a potent DNA-strand breaker (Snyder, et al., (1989). Mutation Res. 226, 185-190; Haaf, 1995). Incubation of cells with the spindle poison colcemid (1 μg/ml for 24 hrs) resulted in the formation of multinuclei and micronuclei containing entire chromosomes. Under the experimental conditions chosen, colcemid does not cause chromosome breakage. Treatment with etoposide

25 (Sedivy), a drug that inhibits DNA topoisomerase II, is a classic system for inducing apoptosis in cells (Mizumoto, et al., (1994). Mol. Pharmac. 46, 890-895).

Antibody Probes. HsRad51 protein, expressed in *E. coli*, was isolated and used for preparation of rabbit polyclonal antibodies. Western blotting experiments revealed

- that rabbit antiserum does not react significantly with any other proteins in mammalian cells except Rad51 (Haaf et al., 1995). Similarly, polydonal antibodies against HsRadS2, a structural homolog of yeast Rad52, were raised in the rat, as is known in the art. Mouse monoclonal antibody 30T14 recognizes Gadd45, a
- 5 ubiquitously expressed mammalian protein that is induced by DNA damage (Smith, et al., (1994). *Science* 266, 1376-1380). Monoclonal antibodies H4 and H14 bind specifically to the large subunit of RNAPII (Bregman et al., (1995) *J. Cell Biol.* 129, 287-298). Monoclonal antibody Pab246 against amino acids 88-93 of mouse p53 was purchased from Santa Cruz Biotechnology, Inc.
- 10 **Immunofluorescent Staining.** Harvested cells were washed and resuspended in PBS. Cell density was adjusted to $\sim 10^5$ cells/ml. 0.5 ml aliquots of this cell suspension were centrifuged onto clean glass slides at 800 rpm for 4 min, using a Shandon Cytospin. Immediately after cyto centrifugation, the preparations were fixed in absolute methanol for 30 min at -20°C and then rinsed in ice-cold acetone for a
- 15 few seconds. Following three washes with PBS, the preparations were incubated at 37°C with rabbit anti-HsRad51 antiserum, diluted 1:100 with PBS, in a humidified incubator for 30 min. For some experiments, the slides were simultaneously labeled with rat anti-HsRad52 antiserum or mouse monoclonal antibody. The slides were then washed in PBS another three times for 10 min each and incubated for 30 min
- 20 with fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit IgG, appropriately diluted with PBS. Rad52, Gadd45, p53, and RNAPII were detected with rhodamine, conjugated anti-rat IgG or anti-mouse IgG+IgM. After three further washes with PBS, the preparations were counterstained with $1\ \mu\text{g/ml}$
- 25 4,6-diamidino-2-phenylindole (DAPI) in 2xSSC for 5 min. The slides were mounted in 90% glycerol, 0.1 M Tris-HCl, pH 8.0, and 2.3% 1,4-diazobicyclo-2,2,2-octane (DABCO).

For preparation of chromatin fibers, cells were centrifuged onto a glass slide and covered with 50 μl of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1% SDS. The protein-extracted chromatin was mechanically sheared on the slide with the aid of

another slide (Heiskanen, et al., (1994) *BioTechniques* 17, 928-933) and then fixed in methanol/acetone.

Fluorescence In Situ End Labeling (FISEL). FISEL detects cell death (apoptosis) in situ by quantitating DNA strand breaks in individual nuclei. It uses terminal transferase (TdT) to label the 3'-ends in fragmented genomic DNA with biotinylated nucleotide. 100 µl of reaction mix contain 1 µl (25 Units) TdT (Boehringer Mannheim), 20 µl 5xTdT buffer (supplied with the enzyme), 1 µl 0.5 mM biotin-11-dUTP, 3 µl 0.5 mM dTTP, and 75 µl ddH₂O. Cytological preparations are incubated at 37°C for 1 hr with this reaction mix. Washing the slides for 3x5 min in PBS is sufficient to terminate the reaction. The incorporated biotin-dUTP is detected with rhodamine-conjugated avidin.

In Situ Labeling of DNA-Replication Synthesis. The base analog BrdU is incorporated in place of thymidine into the DNA of replicating cells. In order to mark cycling cells, 10 µg/ml BrdU were added to the culture medium 30 hrs before cell harvesting. Depending on the cell substrate, this corresponds to one or two population doublings. At the end of the labeling period, slides were prepared as described above. After Rad51-protein staining, the preparations were again fixed in a 3:1 mixture of methanol and acetic acid for several hours at -20°C. Since the anti-BrdU antibody only recognizes BrdU incorporated into chromosomal DNA if the DNA is in the single-stranded form, the slides were denatured in 70% formamide, 2xSSC for 1 min at 80°C and then dehydrated in an alcohol series. BrdU incorporation was visualized by indirect anti-BrdU antibody staining. First, the preparations were incubated with mouse monoclonal anti-BrdU antibody (Boehringer Mannheim), diluted 1:50 with PBS, for 30 min. The slides were washed with PBS and then incubated with rhodamine-conjugated anti-mouse IgG, diluted 1:20 with PBS, for another 30 min. Only cells with intense BrdU labeling of the entire nucleus were considered BrdU-positive and scored as cycling cells.

Overexpression of HsRad51 Protein in Mammalian Cells. Human kidney cells (line 293, ATCC CRL1573) were stably transformed by plasmid pEG9 15. This

plasmid carries the whole coding sequence of the *HsRad51* gene inserted in frame with the 5'-end terminal sequence of vector pEBVHisB (Invitrogen). The resulting cell lines 710 and 717 constitutively express Rad51 protein fused to a T7-tag epitope (Haaf et al., 1995).

- 5 **Digital Imaging Microscopy.** Images were taken with a Zeiss epifluorescence microscope equipped with a thermoelectronically cooled charge coupled device (CCD) camera (Photometries CH250), which was controlled by an Apple Macintosh computer. Gray scale source images were captured separately with filter sets for FITC, rhodamine, and DAPI. Gray scale images were pseudocolored and merged
- 10 using ONCOR Image and ADOPE Photoshop software. It is worth emphasizing that although a CCD imaging system was used, the immunofluorescent signals described here were clearly visible by eye through the microscope.

- Dynamic Nuclear Distribution of Rad51 Protein after DNA Damage** Nuclear foci of mammalian Rad51-recombination protein can be induced significantly after
- 15 irradiation of cell cultures with Cesium (^{137}Cs). Since Western blots have not shown a dramatic net increase in Rad51 protein in irradiated cells, we conclude that DNA damage mainly affects its nuclear distribution (Haaf et al., 1995). To gain insight into the radiation-induced perturbations in nuclear organization and the possible role of Rad51 protein in repair processes, we have analyzed the topological
- 20 rearrangements of Rad51-protein foci in rat TGR-I fibroblasts that have sustained DNA damage. TGR-I is an immortal rat cell line with a stably diploid karyotype. After ^{137}Cs irradiation with a dose of 900 red which kills 99% of cells, rat Rad51 protein was visualized in situ using polyclonal antibodies raised against HsRad51. The percentage of cells with cytologically detectable Rad: 1-protein foci started to
- 25 increase in the first three hours (Table 6). Rad51-positive nuclei contained up to several dozen discrete foci throughout their nucleoplasm. Immunofluorescence staining was largely excluded from the cytoplasm. Many of these nuclear Rad51 foci had a double-dot appearance, typical of paired DNA segments (Figure 3a).

Table 6. Induction of Rad51 Foci after ^{137}Cs Irradiation of TGR-1 cells and Their Elimination into Micronuclei

Treatment	Percentage of Cells without Foci	Percentage of Cells with Type I ^a Foci		Percentage of Cells with Type II ^a Foci	
		in Nuclei	in Micronuclei	in Nuclei	in Micronuclei
None	93%	6%	0%	1%	0%
5 3 hrs after 900 rad ^{137}Cs	80%	8%	0.4%	11%	0.6%
10 16 hrs after 900 rad ^{137}Cs	73%	9%	8%	1%	9%
30 hrs after 900 rad ^{137}Cs	72%	1%	13%	1%	13%
15 4 days after 900 rad ^{137}Cs	90%	0%	4%	0%	6%

^aType I nuclei and micronuclei show weak to medium HsRad51 immunofluorescence, whereas type II cells show strongly fluorescing foci. 1000 cells were analyzed for each experiment.

When irradiated cells were then cultured for various times to allow repair of induced DNA damage and apoptosis to occur, significant changes in the distribution of Rad51-protein foci were detected. Nuclear foci coalesced into larger clusters with extremely high immunofluorescence intensity after 6-20 hrs. Only a few discrete foci remained singly in the nucleoplasm. In a percentage of nuclei linear strings of 5-10 Rad51-protein foci were formed (Figure 3b). Immediately striking was the somatic association of "homologous" strings of similar length. These strings were always tightly paired at one of their ends. The dynamics of the Rad51-protein foci after induction of DNA damage are clear evidence for a higher-order organization of nuclear structure that accompanies DNA repair and/or programmed cell death.

One to two days after ^{137}Cs irradiation with a lethal dose the coalesced Rad51 clusters showed a highly non-random localization towards the nuclear periphery (Figure 3c). Finally, the Rad51 structures were excluded into micronuclei. The nucleoplasm was virtually cleared of Rad51 protein and only aggregated Rad51 foci in MN were remaining (Figure 4; Table 6). Similar to the situation seen earlier in interphase nuclei, many MN displayed paired Rad51 foci and higher-order structures. The highest number of MN (approximately three per cell) as well as the highest number of Rad51-positive MN (approximately 30%) were observed 16 hrs after irradiation (Table 7). However, at each time point analyzed the majority of radiation-induced MN did not show detectable Rad51-protein foci.

Table 7. Rad51 Foci in Micronuclei of Different Cell Substrates

	Cell substrate Treatment	Number of Micronuclei in 1000 Cells	Percentage of Rad51-Positive Micronuclei	Percentage of Rad51-Negative Micronuclei
5	TGR01			
	None	93	14%	86%
	3 hrs after 900 rad ¹³⁷ Cs	279	22%	78%
	16 hrs after 900 rad ¹³⁷ Cs	2719	28%	72%
	10 4 days after 900 rad ¹³⁷ Cs	1040	20%	80%
15	LNL8			
	None	n.d.	23%	77%
	None	n.d.	26%	74%
	XPA			
	None	n.d.	18%	82%
20	Teratoma			
	None	n.d.	10%	90%
	3T3-Swiss			
	None	472	125%	88%

1000 cells were analyzed for each experiment

Segregation of Rad51-Protein Foci into Micronuclei An increased rate of MN is also observed in 5-azadeoxycytidine (5-aza-dC)-treated cell cultures (Guttenbach, et al., (1994) Exp. Cell Res. 211, 127-132; Stopper et al., 1995, supra). This

hypomethylating base analog induces inhibition of chromatin condensation, leading to instability of the affected chromosome regions (Haaf, 1995). Its cytotoxic effects are at least partially due to the induction of single- and double-strand breaks in DNA. Like ^{137}Cs irradiation, 5-aza-dC can induce the formation of Rad51-protein foci in nuclei and its elimination into MN. Rat TGR-1 and human LNL8 fibroblast cultures treated with non-lethal doses of 5-aza-dC displayed MN with focally concentrated Rad51 protein in 5-10% of their cells (Table 8).

Table 8. Induction of Rad51 Foci by 5-Azadeoxycytidine

Cell type Treatment	Percentage of Cells without Foci	Percentage of Cells with Type I ^a Foci		Percentage of Cells with Type II ^a Foci	
		in Nuclei	in Micronuclei	in Nuclei	in Micronuclei
TGR-1					
None	93%	6%	0%	1%	0%
5-aza-dC ^b	86%	5%	4%	1%	4%
LNL8					
None	92%	6%	1%	1%	0%
5-aza-dC ^b	89%	3%	1%	2%	5%

^a Type I nuclei and micronuclei show weak to medium HsRad51 immunofluorescence, whereas type II cells show strongly fluorescing foci. 500 cells were analyzed for each experiment.

^b 10^{-5} M 5-aza-dC were added to the culture medium 24 hrs before cell harvest.

Rapidly dividing cell cultures always exhibit a baseline MN frequency even without exposure to clastogens or aneuploidogens. In five different substrates studied, LNL8, XPA, teratoma, 3T3-Swiss, and TGR-1 cells, 10-30% of these spontaneously occurring, non-induced MN exhibited Rad51-protein foci (Table 7). This further links Rad51-protein foci and MN formation.

Rad52 and Other Repair Proteins Are Not Excluded in Micronuclei Studies
in yeast (Shinohara et al., 1992, supra; Milne, G., and Weaver, D. (1993). *Genes Dev.* 7, 1755-1765) and humans (Shen, et al., (1996). *J. Biol. Chem.* 271, 148-152) have shown physical interaction between Rad51 and Rad52 proteins both in vitro and in vivo. Double immunofluorescence with rabbit anti-Rad51 and rat anti-Rad52 antibodies on ¹³⁷Cs irradiated TGR-1 cells showed that both proteins are enriched in nuclear foci but they do not co-localize. Rad52-protein foci remained in the nucleus throughout the entire time course, while Rad51-protein foci were segregated into MN (data not shown). The same holds true for Gadd45 (data not shown) an inducible DNA-repair protein that is stimulated by p53 (Smith et al., 1994, supra). Biochemical evidence further suggests specific protein-protein association between HsRad51 and p53 (Sturzbecher et al., 1996, supra). However, after anti-p53 antibody staining the Rad51 foci were not particularly enriched with p53 protein (data not shown). In addition, HsRad51 was reported to be associated with a RNA polymerase II (RNAPII) holoenzyme (Maldonado et al., 1996, supra). Although RNAPII was immunolocalized in discrete discrete nuclear foci, as reported previously (Bregman et al., 1995, supra), transcription complexes did not coincide with Rad51 foci (data not shown).

Association of Rad51 Protein with DNA Fibers In a few (<1%) cells of irradiated and drug-treated cultures, we observed very elongated Rad51 structures, up to several hundred micrometer in length, that were eliminated from the nuclei. Since these fiber-like structures stained DAPI-positively, they are thought to contain single DNA molecules of several megabases covered with Rad51 (data not shown). Fluorescence in situ end labeling (FISEL) demonstrated that these DNA fibers contain fragmented DNA typical of apoptosis (data not shown). Sometimes the DNA fibers appeared to leak out of the nucleus through holes in the nuclear membrane and condense into micronuclei. In all cell substrates studied, a high percentage of MN displayed genome fragmentation (data not shown).

The association of Rad51 protein with DNA was also visible on experimentally extended chromatin fibers from irradiated cells. SDS lysis and mechanical stretching

of nuclear chromatin across the surface of a glass slide can cause complete deattachment of DNA loops from the nuclear matrix, producing highly elongated, linear chromatin fibers (Haaf, T., and Ward, D.C. (1994). *Hum. Mol. Genet.* 3, 629-633.; Heiskanen et al., 1994, *supra*). Immunofluorescence staining revealed linear strings of Rad51 label on these stretched DNA fibers (data not shown). By comparison with YAC hybridization signals on similar preparations (Haaf and Ward, 1994, *supra*), the length of the Rad51 fibers was estimated 1-2 Mb.

Rad51-Protein Foci and Apoptosis To determine whether Rad51-positive MN specifically detect exposure to clastogens, analyses were performed in rat TGR-I cells with the aneuploidogen colcemid. This mitotic spindle poison causes lagging of whole chromosomes that are excluded into MN. Surprisingly, when colcemid-treated cells were allowed to recover for 24 hrs in drug-free medium, over 30% of the induced MN contained very brightly fluorescing Rad51 foci (Table 9). Some MN contained rod-like linear structures (data not shown) similar to those observed in Rad51-overexpressing cells. Most of these Rad51-positive MN, 24 hrs after colcemid, did not contain fragmented DNA, as determined by simultaneous FISEL (Table 9). When cells were grown for one or two more days in the absence of the drug, the percentage of Rad51-containing MN decreased dramatically. In addition, the Rad51 protein was no longer concentrated in discrete foci but appeared to disperse throughout the entire MN volume. At the same time most MN became apoptotic and by FISEL their degraded DNA showed incorporation of fluorescent nucleotides. Thus, we conclude that mitotic arrest after colcemid triggers a cascade that induces the elimination of Rad51 protein into MN and drives apoptotic events. Our results seem to be consistent with the hypothesis that apoptosis is a special form of aberrant mitosis (Ucker, D.S. (1991). *New Biologist* 3, 103-1009; Shi et al., 1994, *supra*).

Table 9. Rad51 Foci and Apoptosis in Colcemid-Induced Micronuclei of TGR-1 Cells

Treatment	Number of micro-nuclei in 1000 cells	Percentage of Cells Showing ^a			
		Rad51-/ FISEL-	Rad51+/ FISEL-	Rad 51+/ FISEL+	Rad51-/ FISEL+
None	93	75%	12%	2%	11%
Colcemid ^b	n.d.	85%	6%	0%	9%
1 day of recovery	1293	54%	31%	1%	14%
2 days of recovery	1061	45%	45%	6%	40%
3 days of recovery	769	43%	7%	4%	46%

^aApoptotic cells show fluorescence in situ end labeling (FISEL+), while cells

without genome fragmentation show absence of labeling (FISEL-). "Rad51+" cells with Rad51 foci, "Rad51-+ cells without foci.

^bTGR-1 cells were grown for 24 hrs in medium containing 0.1 µg/ml colcemid to induce micronucleus formation (without inducing DNA damage). 185 of the colcemid-treated cells were arrested at metaphase, 17% showed multinuclei (>10

micronuclei), and 65% had no micronuclei. The cells were then allowed to recover for various times in the absence of the drug. 500 micronuclei were analyzed for each experiment.

Another more classical way for inducing apoptosis in vitro is the exposure of TGR-1 cells to the topoisomerase II inhibitor etoposide. After adding etoposide to the culture medium, the percentage of apoptotic cells steadily increased (Table 10).

After 36 hrs half of the cells showed genome fragmentation and stained FISEL-positively. The nuclear events of apoptosis were accompanied by the appearance of Rad51 protein in nuclei and MN. These results indicate that different stimuli (e.g., irradiation and DNA-damaging drugs, topoisomerase inhibitors, and aneuploidogens) that condemn cells to apoptosis can induce focal concentration of Rad51 protein and its exclusion into MN.

Table 10. Induction of Rad51 Foci and Apoptosis by Etoposide Treatment of TGR-1 Cells

	Treatment	Percentage of Apoptotic Cells ^b	Percentage of Cells without Foci	Percentage of Cells with Type I ^a Foci		Percentage of Cells with Type II ^a Foci	
				in Nuclei	in Micronuclei	in Nuclei	in Micronuclei
	None	6%	93%	6%	0%	1%	0%
5	2 hrs after etoposide ^c	n.d.	92%	4%	1%	1%	2%
	5 hrs after etoposide	n.d.	92%	3%	2%	1%	2%
10	12 hrs after etoposide	17%	87%	8%	2%	1%	2%
	18 hrs after etoposide	24%	79%	3%	8%	1%	9%
	24 hrs after etoposide	33%	82%	2%	2%	6%	8%
15	36 hrs after etoposide	47%	83%	2%	5%	1%	9%

^aType I nuclei and micronuclei show weak to medium HsRad51

immunofluorescence, whereas type II cells show strongly fluorescing foci. 500 cells were analyzed for each experiment.

20 ^bDetected by fluorescence in situ end labeling (FISEL+).

^cCells were grown in medium containing etoposide for the indicated times.

Higher-Order Nuclear Organization of Overexpressed Rad51 Protein Human 293 cells were transfected with the HsRad51 gene. The resulting cell lines 710 and 717 constitutively expressed a HsRad51-fusion protein. This overexpressed protein 25 formed brightly fluorescing linear structures inside the nucleus (Figure 7a). Some nuclei were completely filled with a network of rod-like structures (Figure 7b). Identical Rad51 structures were observed in transformed rat TGR 928.1-9 cells, stably expressing the HsRad51 protein without a tag epitope (data not shown). This suggests that Rad51 protein is able to assemble into higher-order structures within 30 the highly ordered interphase nucleus. The linear nature of Rad51 structures in

overexpressing cells is reminiscent of the strings of Rad51-protein foci after DNA damage and colcemid treatment and in meiotic cells (Haaf et al., 1995).

However, in contrast to the situation after DNA damage, the overexpressed HsRad51 protein is not eliminated into MN. The numbers of Rad51-positive MN were not
5 radically different in Rad51-overexpressing human 717 cells versus in 293 control cells and in rat 928.1-9 overexpressers versus in parental TGR-1 cells. This means that Rad51 overexpression alone does not cause apoptosis. In exponentially growing unsynchronized cultures, 14% of both 717 and 293 cells (500 cells were analyzed for each experiment) and 8% of both 928.1-9 and TGR-1 cells showed
10 cleavage of the cell's DNA by FISEL. We conclude that the segregation of Rad51 into MN is a specific behavior of apoptotic cells and precedes genome fragmentation.

Cell-Cycle Arrest of Cells with Focally Concentrated RadS1 Protein

Simultaneous Rad51-protein immunofluorescence and antibromodeoxyuridine
15 (BrdU) antibody staining demonstrated that nuclei with focally concentrated Rad51 protein do not undergo DNA-replication synthesis (data not shown). BrdU was incorporated into replicating DNA of unsynchronized cell cultures for 30 hrs. Rapidly growing transformed cell lines (293, LNL8, XPA, and XPF) which showed detectable Rad51 immunolabelling in a percentage of nuclei even without induction of
20 DNA damage as well as Rad51 overproducers (928.1-9 and 717) were analyzed. For each experiment, 100 nuclei with prominent Rad51 foci and 100 nuclei without detectable Rad51 foci were stained with fluorescent anti-BrdU antibody. In the widely different substrates tested, 80%-100% of the cells with focally concentrated Rad51 protein were found to be BrdU-staining negative (Table 11). In contrast,
25 30%-90% of the cells without Rad51 foci from the same cultures showed BrdU incorporation, indicative of cycling cells. The BrdU-substituted DNA was located in discrete replication sites throughout the nucleus as reported previously (Nakayasu, H., and Berezney, R. (1989). *J. Cell Biol.* 108, 1-11; Fox, et al., (1991) *J. Cell Sci.* 99, 247-253). This suggests that even without induction of DNA damage the cells

with Rad51 foci are arrested during the cell cycle or enter S phase delayed of the Rad51-foci negative cells.

Table 11 Induction of Rad51 Foci after ^{137}Cs Irradiation of TGR-1 cells and Their Elimination into Micronuclei

Treatment	Percentage of Cells without Foci	Percentage of Cells with Type I ^a Foci		Percentage of Cells with Type II ^a Foci	
		in Nuclei	in Micronuclei	in Nuclei	in Micronuclei
None	93%	6%	0%	1%	0%
3 hrs after 900 rad ^{137}Cs	80%	8%	0.4%	11%	0.6%
16 hrs after 900 rad ^{137}Cs	73%	9%	8%	1%	9%
30 hrs after 900 rad ^{137}Cs	72%	1%	13%	1%	13%
4 days after 900 rad ^{137}Cs	90%	0%	4%	0%	6%

^aType I nuclei and micronuclei show weak to medium HsRad51 immunofluorescence, whereas type II cells show strongly fluorescing foci. 1000 cells were analyzed for each experiment.

Rat TGR-1 cells are capable of normal physiological withdrawal into the quiescent (Go) phase of the cell cycle as well as resumption of growth following the appropriate stimuli (Prouty, et al., (1993). Oncogene 8, 899-907). In TGR 928.1-9 cells overexpressing a HsRad51 transgene(s), Go arrest upon serum starvation dramatically induced HsRad: 1-protein foci (Table 12). Synchronous re-entry into the cell cycle after feeding reduced the percentage of HsRad51-foci positive cells to very low levels. However, new Go arrest upon contact inhibition following three

population doublings increased the number of cells with nuclear Rad51 foci again.

We therefore conclude that cells with prominent nuclear Rad51 foci are most likely in Go or G1 phase of the cell cycle.

Table 12. Rad51 Foci in Micronuclei of Different Cell Substrates

5	Cell substrate Treatment	Number of Micronuclei in 1000 Cells	Percentage of Rad51-Positive Micronuclei	Percentage of Rad51-Negative Micronuclei
	TGR01			
	None	93	14%	86%
10	3 hrs after 900 rad ¹³⁷ Cs	279	22%	78%
	16 hrs after 900 rad ¹³⁷ Cs	2719	28%	72%
	4 days after 900 rad ¹³⁷ Cs	1040	20%	80%
15	LNL8			
	None	n.d.	23%	77%
	None	n.d.	26%	74%
	XPA			
	None	n.d.	18%	82%
20	Teratoma			
	None	n.d.	10%	90%
	3T3-Swiss			
	None	472	125%	88%

25 1000 cells were analyzed for each experiment

Other references specifically incorporated by reference are Haaf, T. (1995).

Pharmac. Ther. 65, 19-46; Haaf, T., and Schmid, M. (1991). Exp. Cell Res. 192, 325-332; and Owaga, et al, (1993) Science 259, 1896-1899

CLAIMS

We claim:

1. A method of diagnosing individuals at risk for a disease state comprising
 - a) determining the distribution of Rad51 foci in a first tissue type of a first individual; and
 - b) comparing said distribution to the distribution of Rad51 foci from a second normal tissue type from said first individual or a second unaffected individual;wherein a difference in said distributions indicates that the first individual is at risk for a disease state which results in aberrant Rad51 loci.
2. A method according to claim 1 wherein said disease state is cancer.
3. A method of diagnosing individuals at risk for cancer comprising
 - a) determining the distribution of Rad51 foci in a potential cancerous tissue type of a first individual; and
 - b) comparing said distribution to the distribution of Rad51 foci from a second normal tissue type from said first individual or a second unaffected individual;wherein a difference in said distributions indicates that the first individual is at risk for a cancer which results in aberrant Rad51 loci.
4. A method according to claim 3 wherein the cancer is selected from breast cancer and skin cancer.
5. A method of diagnosing individuals at risk for a disease state associated with apoptosis, said method comprising
 - a) determining the distribution of Rad51 foci in a first tissue type of a first individual; and
 - b) comparing said distribution to the distribution of Rad51 foci from a second normal tissue type from said first individual or a second unaffected individual;wherein a difference in said distributions indicates that the first individual is at risk for a disease state associated with apoptosis which results in aberrant Rad51 loci.

6. A method according to claim 1 wherein the extent of aberrant distribution indicates the severity of the disease state.
7. A method according to claim 1 wherein said distribution is determined through the use of polyclonal antibodies.
- 5 8. A method according to claim 1 wherein said distribution is determined through the use of monoclonal antibodies.
9. A method according to claim 7 or 8 wherein said antibodies are raised against eukaryotic Rad51.
- 10 10. A method according to claim 9 wherein said eukaryotic Rad51 is mammalian Rad51.
11. A method for identifying an apoptotic cell comprising
 - a) determining the distribution of Rad51 foci in a first cell; and
 - b) comparing said distribution to the distribution of Rad51 foci from a second non-apoptotic cell;
- 15 wherein a difference in said distributions indicates that the first cell is apoptotic.
12. A method according to claim 11 wherein said distribution is the association of Rad51 with DNA fibers.
13. A method according to claim 11 wherein said distribution is the association of Rad51 into micronuclei.
- 20 14. A method for identifying a cell under stress associated with nucleic acid modification comprising
 - a) determining the distribution of Rad51 foci in a first cell; and
 - b) comparing said distribution to the distribution of Rad51 foci from a second non-affected cell;

wherein a difference in said distributions indicates that the first cell is under stress associated with nucleic acid modification.

15. A method according to claim 14 wherein said stress is oxidative or hypoxic stress.

5 16. A method according to claim 14 wherein said stress is heat shock.

17. A method according to claim 14 wherein said stress is cold shock.

18. A method for identifying a cell containing a mutant Rad51 gene comprising determining the sequence of all or part of at least one of the endogenous Rad51 genes.

10 19. A method of identifying the Rad51 genotype of an individual comprising determining all or part of the sequence of at least one Rad51 gene of said individual.

20. A method according to claim 18 or 19 further comprising comparing the sequence of said Rad51 gene to a known Rad51 gene.

21. A method according to claim 20 wherein a difference in the sequence between
15 the Rad51 gene of said individual and said known Rad51 gene is indicative of a disease state or a propensity for a disease state.

22. A method for screening for a bioactive agent capable of binding to Rad51 comprising:

- 20 a) adding a candidate bioactive agent to a sample of Rad51; and
b) determining the binding of said candidate agent to said Rad51.

23. A method for screening for a bioactive agent capable of modulating the activity of Rad51, said method comprising the steps of:

- a) adding a candidate bioactive agent to a sample of Rad51; and

b) determining an alteration in the biological activity of Rad51.

24. A method according to claim 23 wherein said biological activity is DNA dependent ATPase activity.

25. A method according to claim 23 wherein said biological activity is nucleic acid
5 strand exchange.

26. A method according to claim 23 wherein said biological activity is DNA binding.

27. A method according to claim 23 wherein said biological activity is filament formation.

10 28. A method according to claim 23 wherein said biological activity is DNA pairing.

29. A method for screening for a bioactive agent capable of modulating the activity of Rad51, said method comprising the steps of:

a) adding a candidate bioactive agent to a cell; and
b) determining the effect on the formation or distribution of Rad51 foci in said
15 cell.

30. A method according to claim 25 further comprising subjecting said cell to conditions which induce nucleic acid damage.

31. A method of inducing apoptosis in a cell comprising increasing the activity of Rad51 in said cell.

20 32. A method according to claim 31 wherein said increasing comprises overexpression of endogenous Rad51.

33. A method according to claim 31 wherein said increasing comprises administration of a gene encoding Rad51.
34. A method according to claim 31 wherein said increasing comprises administration of Rad51 protein.
- 5 35. A method according to claim 31 wherein said cell is a cancer cell.
36. A method according to claim 31 further comprising subjecting said cell to conditions which induce nucleic acid damage.
37. A method according to claim 36 wherein said conditions comprise the administration of a chemical agent which causes nucleic acid damage.
- 10 38. A method according to claim 36 wherein said conditions comprise subjecting said cell to radiation.
39. A method according to claim 31 further comprising increasing the activity of p53 in said cell.
40. A composition comprising:
- 15 a) nucleic acid encoding a Rad51 protein; and
 b) nucleic acid encoding a tumor suppressor protein.
41. A composition according to claim 38 wherein said tumor suppressor protein is p53.
42. A composition according to claim 38 wherein said tumor suppressor protein is
- 20 BRCA1.
43. A composition according to claim 38 wherein said tumor suppressor protein is BRCA2.

44. A composition according to claim 38 comprising:
- a) nucleic acid encoding a Rad51 protein;
 - b) nucleic acid encoding a BRCA1 protein;
 - c) nucleic acid encoding a BRCA2 protein; and
 - 5 d) nucleic acid encoding a p53 protein.
45. A composition comprising:
- a) a recombinant Rad51 protein; and
 - b) a recombinant tumor suppressor protein.
46. A kit for detecting the distribution of Rad51 foci in a cell or tissue comprising:
- 10 a) binding agent for Rad51 foci; and
 - b) a detectable label.

Types of Rad51 Foci

Type II

Type I

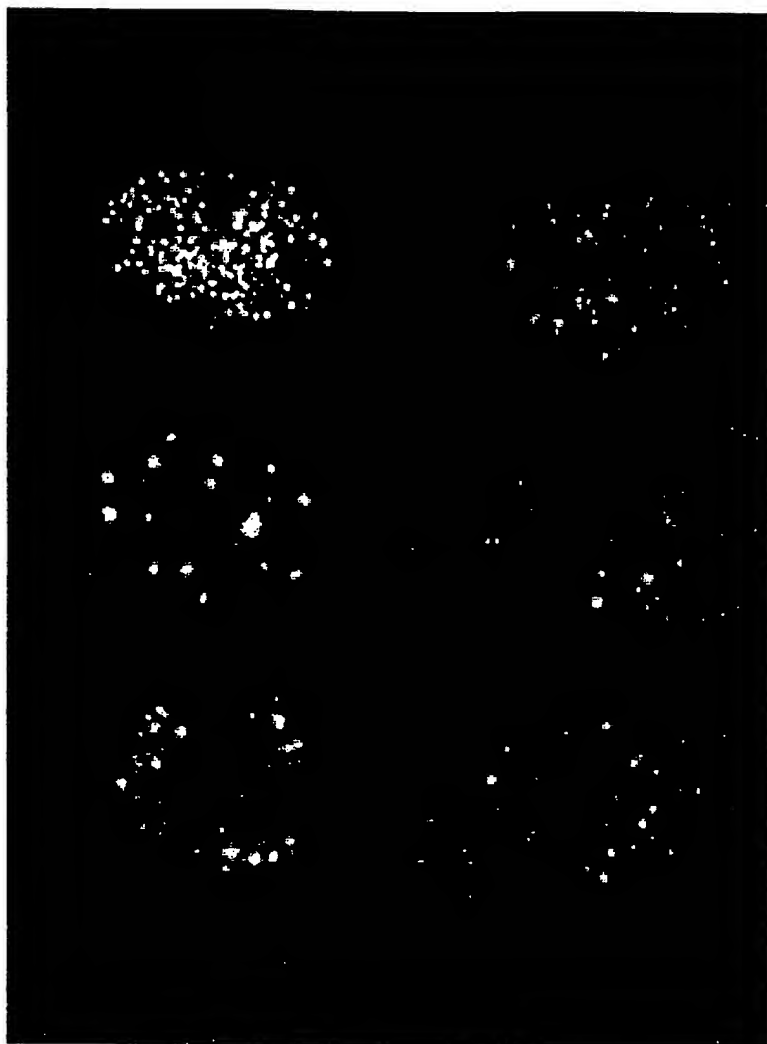


FIG. 1

FIG. 2A



FIG. 2B

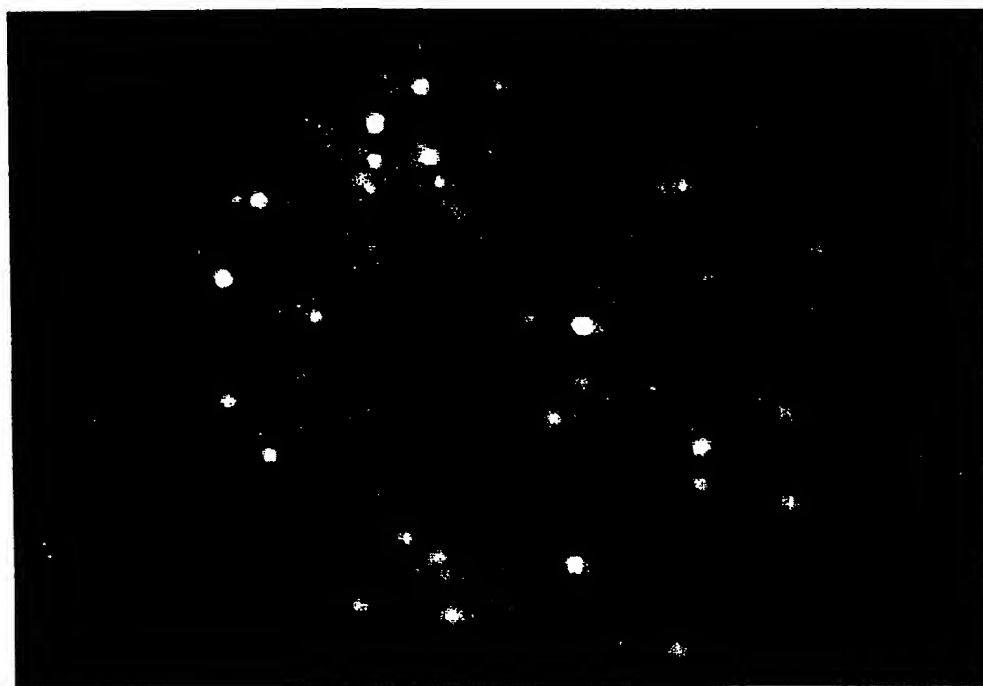
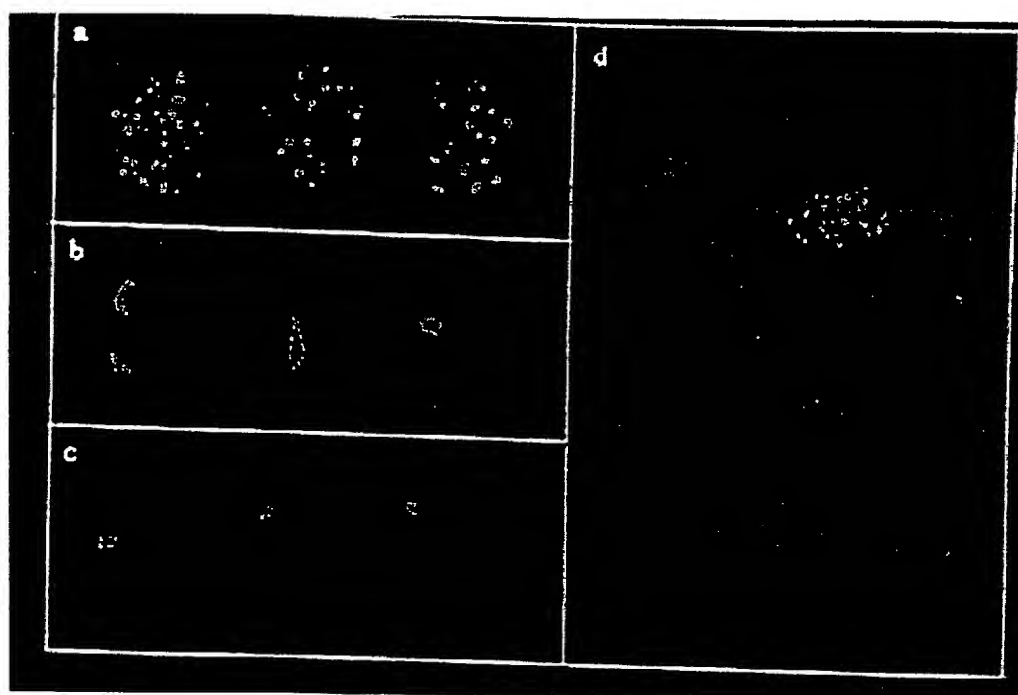


FIG. 3A

FIG 3D

IG. 3B

IG. 3C



5/8
FIG. 4

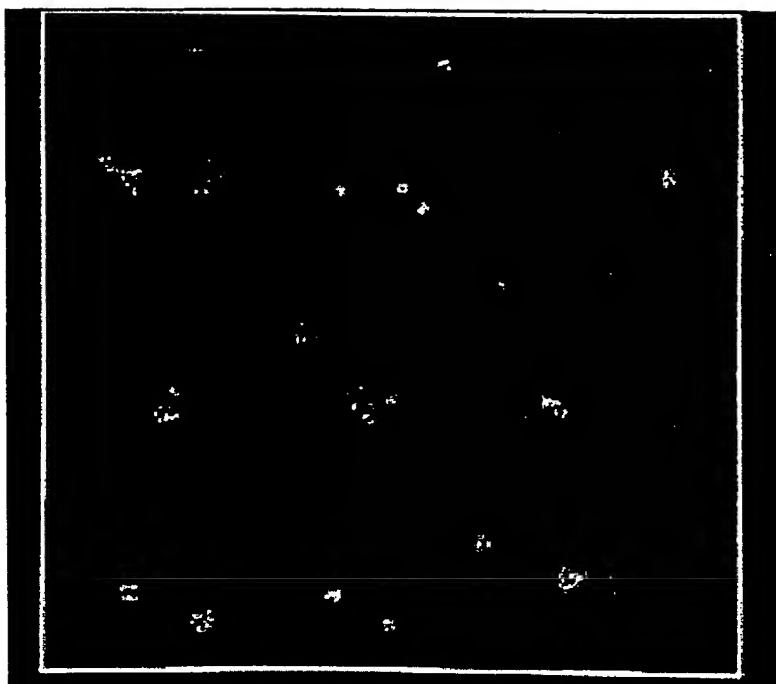
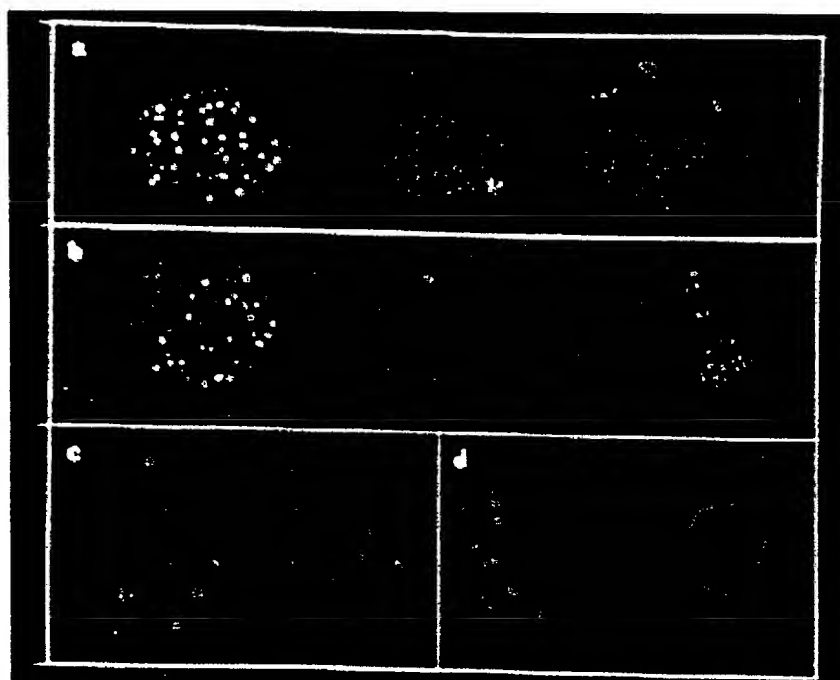


FIG. 5A

FIG. 5B

FIG. 5C

FIG. 5D



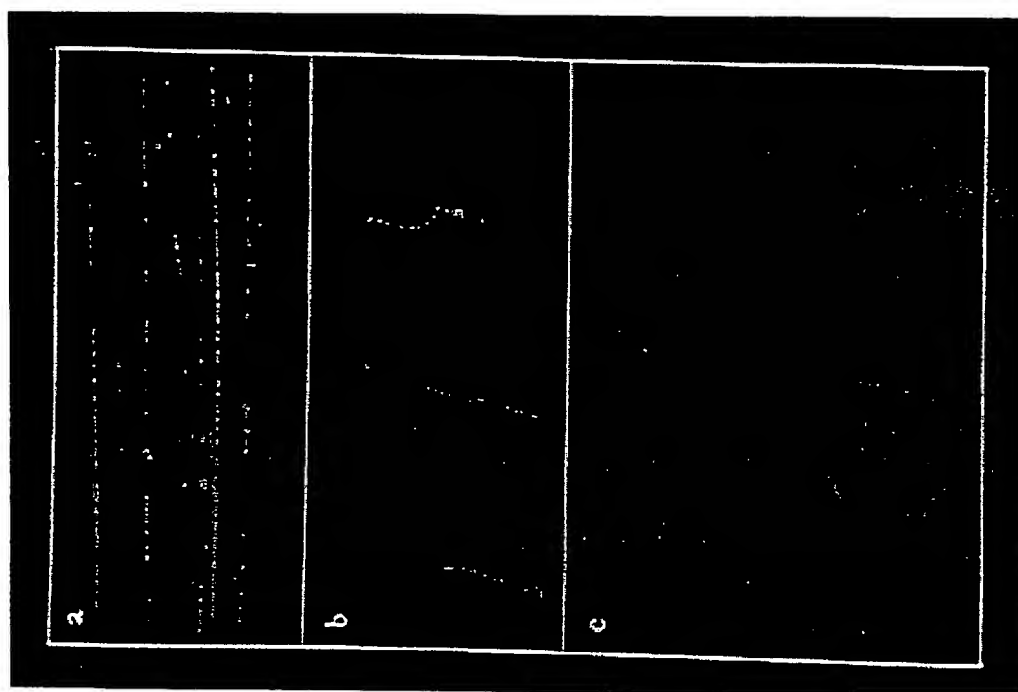
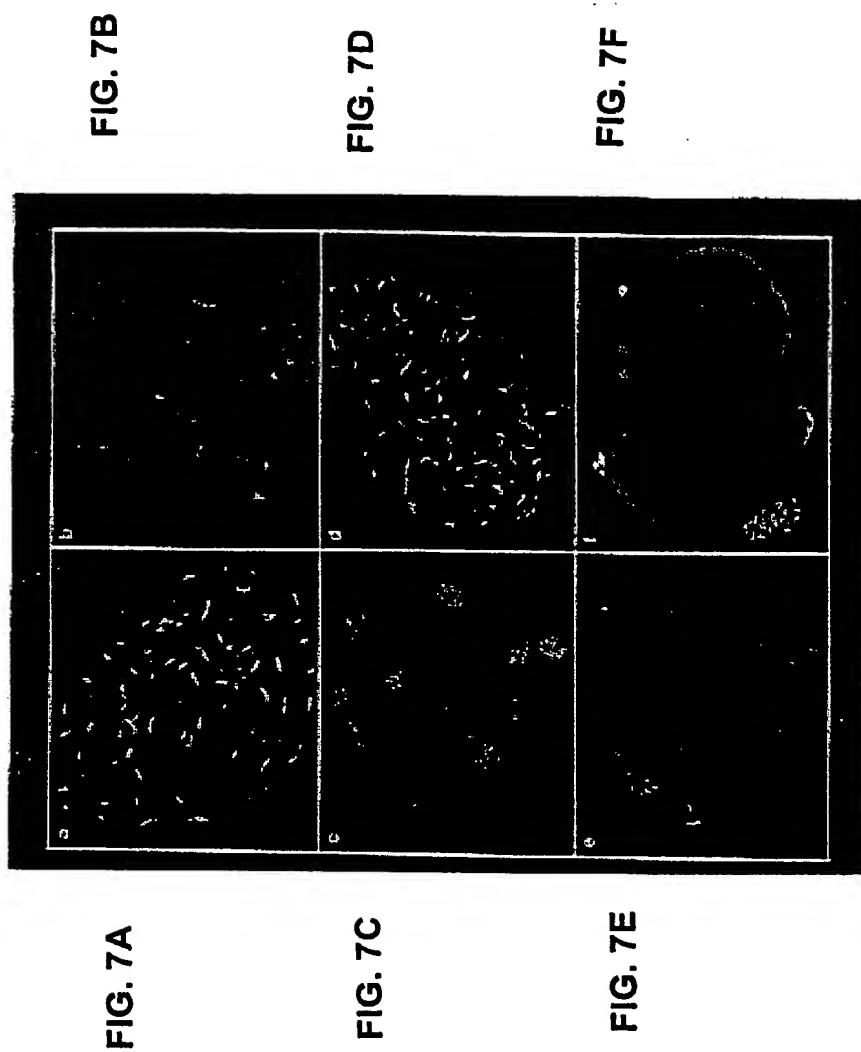


FIG. 6A

FIG. 6B

FIG. 6C



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/01825

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/574 G01N33/50 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHANET, R. ET AL.: "Semidominant mutations in the yeast RAD51 protein and their relationships with the Srs2 helicase." MOLECULAR AND CELLULAR BIOLOGY, vol. 16, no. 9, 1996, pages 4782-4789, XP002065424 see the whole document	18-21
X	STÜRZBECHER, H-W. ET AL.: "p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction" THE EMBO JOURNAL, vol. 15, no. 8, 1996, pages 1992-2002, XP002065425 see the whole document	22-30
X	see figure 1	45

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

25 May 1998

Date of mailing of the international search report

19. 06. 1998

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/01825

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>SHARAN, S.K. ET AL.: "Embryonic lethality and radiation hypersensitivity mediated by RAD51 in mice lacking Brca2"</p> <p>NATURE, vol. 386, 24 April 1997, pages 804-810, XP002065426 see the whole document</p> <p>-----</p>	1-46

INTERNATIONAL SEARCH REPORT

I. national application No.
PCT/US 98/01825

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 31-39
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 31-39 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the RAD51 gene or protein.

Claims Nos.: 31-39

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

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Summary

<u>Document</u>	<u>Pages</u>	<u>Printed</u>	<u>Missed</u>	<u>Copies</u>
WO009834118	78	78	0	1
Total (1)	78	78	0	-

Methods in Enzymology

Volume 93

IMMUNOCHEMICAL TECHNIQUES

Part F

Conventional Antibodies,
Fc Receptors, and Cytotoxicity

sensitive for situations in which very few cytotoxic cells are available, or the effector cell is not testable by other current methods. In addition, the single-cell assay provides a new means to examine the biological and biochemical mechanisms of the cytotoxic reactions at the level of individual cells.

Acknowledgment

The work reported here was supported by Grants CA-12800, CA-19753, and CA-24314 from the National Cancer Institute, U.S. Public Health Service.

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[20] Preparation of Antibody-Linked Cytotoxic Agents*

By TARUN I. GHOSE, A. HUNTLEY BLAIR, and PADMAJA N. KULKARNI

The purpose of linking cytotoxic agents to antibodies against cell surface antigens is to direct an agent selectively to the antigen-bearing cell population.¹ This approach to targeting has been used chiefly in experimental models for inhibition or destruction of cancer cells and T lymphocytes. Agents that have been linked to immunoglobulins include simple radionuclides, cancer chemotherapeutic drugs, and toxins of microbial and plant origin.^{1,2} In this chapter we shall discuss the strategy of linking cytotoxic agents to immunoglobulins so that biologically active conjugates retaining agent and antibody activities are produced. Methods that have been used or are potentially useful for linkage will be described. Linkage of radionuclides will not be covered, since this subject has been reviewed recently.^{2,3} Several recent publications may be consulted on the

* Reference numbers beginning with V and VI denote references in Tables V and VI, respectively.

¹ T. Ghose and A. H. Blair, *J. Natl. Cancer Inst.* **61**, 657 (1978).

² T. Ghose, A. H. Blair, P. Kulkarni, K. Vaughan, S. T. Norvell, and P. Belitsky, in "Targeting of Drugs" (G. Gregoriadis, ed.), p. 55. Plenum, New York, 1982.

³ T. Ghose, A. H. Blair, R. H. Martin, S. T. Norvel, S. Ramakrishnan, P. Belitsky, K. Imai, and S. Ferrone, in "Tumor Imaging" (S. W. Burchiel, B. A. Rhodes, and B. E. Friedman, eds.), p. 167. Masson, Paris, 1982.

binding of drugs and haptens to macromolecular carriers,⁴⁻¹¹ and linkage of enzymes and toxins to antibodies.^{12,13}

Strategy for Linkage of Cytotoxic Agents to Immunoglobulins

For antibody-mediated selective delivery of chemotherapeutic agents or toxins an ideal conjugation method should (a) not interfere with either the agent or antibody activity; (b) allow high incorporation, yet be sufficiently controllable to be tailored to the production of homogeneous conjugates containing optimal agent to antibody ratios; (c) avoid formation of homopolymers of antibody or agent; (d) avoid aggregation of the conjugate; and (e) be technically straightforward and reproducible. Further, the drug or toxin must be delivered to the milieu of the sensitive target molecules in a form that is active or can be activated *in situ*. It is to be noted that a conjugated cytotoxic agent may damage target cells by its interaction with alternative or altogether different target molecules from those susceptible to the action of free drug. For example, it has been postulated that Ig-linked Trenimon, unlike the free drug, is neither internalized nor alkylates nuclear DNA; its inhibitory effect appears to involve alterations in the plasma membrane.¹⁴⁻¹⁹

Selection of a method for linkage of a cytotoxic agent to immunoglobulins should be based on the following points.

1. Consideration of the type of bond required for optimal biological action, e.g., one that will permit cytotoxic action in the intracellular milieu either by maintaining a favorable steric orientation or allowing release of active agent.
2. Identification of existing reactive groups in the agent and Ig that are not essential for activity. However, an essential group might be used for linkage if the bond formed was one that could be cleaved

⁴ B. F. Erlanger, this series, Vol. 70, p. 85.

⁵ G. Gregoriadis, in "Drug Carriers in Biology and Medicine" (G. Gregoriadis, ed.), p. 1. Academic Press, New York, 1979.

⁶ L. Gros, H. Ringsdorf, and H. Schupp, *Angew. Chem. Int. Ed. Engl.* **20**, 305 (1981).

⁷ E. A. Kabat, in "Structural Concepts in Immunology and Immunochemistry" (E. A. Kabat, ed.), p. 28. Holt, New York, 1976.

⁸ S. B. Kadin and I. G. Otterness, *Annu. Rep. Med. Chem.* **15**, 233 (1980).

⁹ S. W. Kim, R. V. Petersen, and J. Feijen, in "Drug Design" (E. J. Ariens, ed.), Vol. 10, p. 193. Academic Press, New York, 1980.

¹⁰ M. J. Pozansky and L. G. Cleland, in "Drug Delivery Systems" (R. L. Juliano, ed.), p. 253. Oxford Univ. Press, London and New York, 1980.

¹¹ D. S. Zaharko, M. Przybylski, and V. T. Oliverio, *Methods Cancer Res.* **16**, 347 (1979).

¹² S. Avrameas, T. Ternynck, and J. L. Guesdon, *Scand. J. Immunol.* **8**, Suppl. 7, 7 (1978).

¹³ M. J. O'Sullivan and V. Marks, this series, Vol. 73, p. 147.

TABLE I
LINKAGES BETWEEN CYTOTOXIC AGENTS AND IMMUNOGLOBULINS

Covalent
Direct
Bridged via intermediaries and spacers such as dextran and polyamino acids
Noncovalent
Ionic bonds, H bonds, and weak interactions between dipoles (i.e., van der Waals/London-type bonds)

intracellularly to regenerate the original group or a derivative of it that allowed cytotoxic action.

3. Assessment of whether the best method of linkage can be achieved by using existing groups or by introducing special reactive groups into agent and/or Ig, e.g., sulfhydryl groups by reaction with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP, see below).
4. Assessment of necessity for additional modifications of the agent or immunoglobulin for production of biologically effective conjugates, such as elimination of the cell surface binding subunit (B chain) of diphtheria toxin or the Fc fragment of IgG (see Table VI).
5. Assessment of the necessity of using intermediaries or spacers to permit increased loading of agent or to provide a favorable steric orientation.
6. Assessment of possible adverse effects of proposed chemical procedures on the activities of agent and antibody or on the biological half-life of the conjugate.

Table I summarizes the possible kinds of linkages between cytotoxic agents and Ig. Even without formation of a covalent bond, the concerted effects of ionic bonds, hydrogen bonds, and van der Waals type of attractions may yield very stable complexes.^{11,14} If action of the agent needs dissociation from the carrier antibody, binding by such noncovalent bonds would be a useful method provided that the binding is stable enough to prevent rapid dissociation of agent in the blood and/or other body fluids.⁶

Structure of Immunoglobulins in Relation to the Linkage of Cytotoxic Agents

Immunoglobulins share a number of distinctive structural features while exhibiting enormous structural diversity compared to other biologi-

¹⁴ A. Goldstein, L. Aronow, and S. M. Kalman, in "Principles of Drug Action: The Basis of Pharmacology" (A. Goldstein, L. Aronow, and S. Kalman, eds.), p. 15. Wiley, New York, 1974.

cally active proteins. Distinctive features include (a) a tetrameric arrangement of two light (L) chains (M_r 23,000) and two heavy (H) chains (M_r 50,000–70,000) linked by disulfide bridges; and (b) the division of each dimer made up of one L and one H chain into variable (V) (antigen binding) and constant (C) regions. The V region occurs near the amino terminal end of the immunoglobulin chain. Immunoglobulins of different classes differ considerably in the amino acid sequence of the C segment of their H chains, in chain length, in the number of homologous domains in the chain, in the disulfide bridge pattern, in the degree of polymerization, and in the position, numbers, and kind of oligosaccharides. When L or H chains from the same class and species are compared, the C region is found to contain an invariant sequence characteristic of the class and species, whereas the V region shows multiple substitutions ranging from 10 to 60 in the first 110 residues. In fact, although several hundred V regions of the two types of L chains, (kappa (κ) and lambda (λ), have been partially or completely sequenced, no two have yet been shown to be identical in amino acid sequence. However, there is an overall homology in amino acid sequence among L chains of the order of 25–35%. The κ and λ L chains have about 40% identity in amino acid sequence, whereas L and H chains share about 25% identity in sequence.

The identification of repeating homologous regions or domains in immunoglobulin molecules has contributed to the understanding of the structural basis of their function. Domains are made up of 110–120 amino acid residues, contain an intrachain disulfide bridge linking some 60 residues, are easily cleaved by proteolytic agents, and have distinctive biological functions associated with them. The variable domains of the L and H chains interact to form the variable fragment, which contains the antigen binding site. Its specificity is determined by the three hypervariable segments of the V regions of both L and H chains. Most of the V domain structure can be regarded as consisting of an invariant framework of antiparallel β -pleated sheet. Edmundson *et al.*¹⁵ have investigated the individual residues in the β -pleated sheets of V and C regions. In V domains the most common polar residues in the alternating patterns of the β -pleated sheets are serine and threonine. The cylindrical surfaces are mainly composed of the side chains of these hydroxylic amino acids, whereas in the C domains, the surface residues are more diversified. There are about 30 hydrophobic side chains in the interior of each V and C domain. The hypervariable segments form connecting loops or hairpin bends at the C domain. They vary widely in higher order as well as primary structure. There appears to be an enormous range of substitu-

¹⁵ A. B. Edmundson, K. R. Ely, E. E. Abola, M. Schiffer, and N. Panagiotopoulos, *Biochemistry* **14**, 3953 (1975).

TABLE II
AMINO ACID RESIDUES AVAILABLE FOR LINKAGE IN IMMUNOGLOBULINS

Nucleophilicity (in descending order)	Functional group	Amino acid
1. Sulfur	Thiol	Cysteine
	Thioether	Methionine
2. Nitrogen	ϵ -Amino	Lysine
	Imidazole	Histidine
	Guanidino	Arginine
3. Oxygen	Carboxyl	Glutamic acid
		Asparatic acid
	Hydroxyl	Serine
		Threonine
	Phenolic hydroxyl	Tyrosine
4. Electron-rich centers	Phenolic ring (positions 3 and 5)	Tyrosine
	Imidazole	Histidine

tions possible while still preserving the framework conformation of the variable domains. Available data on the structure of binding sites are restricted largely to those specific for various haptens in which the amino acid residues tend to be aromatic but interspersed with polar residues.^{7,16,17}

Few proteins have been analyzed so extensively by amino acid sequencing as immunoglobulins or their fragments of both human and animal origin. Atlases of immunoglobulin structure and computer printouts of emerging data on V region sequences are now available as well as the results of a number of X-ray crystallographic studies.^{7,16,17} Table II lists amino acid residues in immunoglobulins that could be used for linkage reactions.

The structural information available on immunoglobulins has not identified particular amino acid residues that might be exploited for conjugation on the basis that they occur solely in the C region, with the exception of the interchain disulfide bridges of cystine. Furthermore, side chains of residues in the antigen binding site are highly accessible because this site is fully exposed to the solvent interface.

In contrast to immunoglobulin amino acid residues, the carbohydrate prosthetic groups may furnish potential linkage sites that will not interfere with antibody activity. Most immunoglobulins contain carbohydrates, but

¹⁶ D. Givol, *Int. Rev. Biochem.* **23**, 71 (1979).

¹⁷ F. W. Putnam, in "The Plasma Proteins" (F. W. Putnam, ed.), 2nd ed., Vol. 3, p. 1. Academic Press, New York, 1977.

these carbohydrates are not involved in antigen binding. They are characteristically present in the C regions of heavy chains and are only occasionally found in the V region (owing to the presence of the obligatory sequence Asn-X-Thr-Ser).^{16,17} Thus carbohydrates offer potential sites for linkage of agents to immunoglobulins without disrupting antigen binding by that antibody.

The total carbohydrate in immunoglobulins varies from 3% in IgG to 13% in IgE. The number, location, and type of oligosaccharides differ in the five classes of human heavy chains. Because of their solubility, hydrophilicity, and bulk, oligosaccharides are found on the exterior of the protein and are thus accessible. These carbohydrates may be either simple (i.e., contain only mannose or *N*-acetylglucosamine) or complex and highly branched, containing smaller amounts of mannose, but galactose, fucose, and sialic acid in addition. Details of the structure of carbohydrate groups in human immunoglobulins can be found in several publications.^{7,18} To assess the availability of these carbohydrate moieties in IgG, we oxidized a rabbit anti-BSA IgG with periodate to form aldehyde groups from vicinal diols in carbohydrate moieties. Reaction with ethylenediamine and sodium borohydride led to the incorporation of 42 additional primary amino groups in the IgG. Seventy-five percent of its antigen binding activity was retained.

Structure of Cytotoxic Agents in Relation to Linkage to Immunoglobulins

Low Molecular Weight Cancer Chemotherapeutic Agents

Low molecular weight cancer chemotherapeutic agents generally provide few (if any) reactive groups that can be modified or used for conjugation without jeopardizing drug activity. For example, the highly reactive groups of alkylating agents must be protected to obtain active conjugates.^{V-11} The pteridine moiety (with its amino groups in position 4) of methotrexate (MTX) must be preserved to obtain MTX-immunoglobulin conjugates that inhibit dihydrofolate reductase (DHFR) and retain cytotoxic activity. Thus, the glutamate moiety at the other end of the molecule has been mostly utilized for modification of this drug and for its conjugation to immunoglobulins and other carriers.^{V-6} For agents, such as the anthracycline glucosides adriamycin and daunomycin, that act by intercalation with DNA, use of bonds that can be cleaved intracellularly to release drug with appropriate steric properties is important for the pro-

¹⁸ A. L. Tarentino, T. H. Plummer, and F. Maley, *Biochemistry* **14**, 5516 (1975).

duction of active drug-antibody conjugates.^{V-30} Because it is the aglycon part of the drug that intercalates with DNA and the amino sugar and a portion of the chromophore remain externalized, substitution at these sites is possible provided that the appropriate charge and the planar lipophilic configuration of the polycyclic ring are retained.¹⁹ Thus the pronounced loss of activity of *N*-acyl²⁰ or *O*-methyl²¹ derivatives of daunomycin is thought to be due to loss of basic character and consequent loss of electrostatic bond formation. However, it should be stated that several mechanisms of antitumor action unrelated to DNA intercalation have been proposed, e.g., inhibition of mitochondrial respiration, alteration of cell surface architecture, and interference with microtubular function.¹⁹

Thus it will be useful to consider the results of studies on structure-action relationships for an agent to identify groups that are suitable for linkage. This may also be of help in assessing the stability of the agent during proposed chemical manipulations. Table III lists commonly used linkage methods that can be applied to various reactive group(s) occurring in cytotoxic agents. See Table V for those low molecular weight cytotoxic agents that have been linked to immunoglobulins and an outline of the method used in each case.

Protein Agents: Plant and Microbial Toxins and Other Enzymes

The extreme lethality of certain bacterial and plant toxins make them attractive candidates for antibody-directed delivery to cancer cells.^{1,2,22} A group of these represented by diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, abrin, ricin, and modeccin consist of two disulfide linked polypeptide chains, i.e., an enzymically active polypeptide (A chain) and a receptor-specific binding polypeptide (B chain).

Single-chain toxin-related proteins form another group of potential toxins that may be useful for linkage to specific carriers. They resemble the A chain of the above group of toxins and by themselves are not toxic to cells, as they lack the B chain and are therefore not internalized. Gelonin, a representative member of this group, has been shown to be toxic to cells when linked through a disulfide bond to concanavalin A²³ and antibody against Thy 1.1 antigen.^{VI-13} A number of other enzymes, such as L-asparaginase and phospholipase C, have been linked either to

¹⁹ H. S. Schwartz, *Adv. Cancer Chemother.* **6**, 11 (1979).

²⁰ D. W. Henry, in "Cancer Chemotherapy" (A. C. Sartorelli, ed.), p. 15. Am. Chemical Society, Washington, D.C., 1976.

²¹ F. Zunino, A. M. Casazza, G. Prates, F. Formelli, and A. Dimarco, *Biochem. Pharmacol.* **30**, 1856 (1981).

²² S. Olsnes, *Nature (London)* **290**, 84 (1981).

²³ F. Stripe, S. Olsnes, and A. Pihl, *J. Biol. Chem.* **255**, 6947 (1980).

TABLE III
REACTIVE GROUPS IN AGENTS AND LINKAGE METHODS UTILIZING THEM

Reactive group	Linkage method ^a	Reactive group in immunoglobulin
Carboxyl (e.g., methotrexate, chlorambucil)	Mixed-anhydride intermediate Carbodiimides Azide	Amino Amino Amino
Amino (aromatic)	Active ester intermediate	Amino
Amino (aliphatic) (e.g., adriamycin, daunomycin)	Diazotization Carbodiimides Dialdehydes	Phenol, indole, imidazole, amino Carboxyl Amino
Hydroxyl (e.g., various steroids, 3-hydroxyclozapepam, 1- β -D-arabinofuranosylcytosine)	Conversion to a <i>p</i> -nitrobenzoylamide followed by reduction and diazotization Reaction with succinic anhydride to form hemisuccinates, then linkage as for carboxyl group	Phenol, indole, imidazole, amino Amino
Phenol	Chlorocarbonates (generated by reaction with phosgene) Oxidation to carboxyl, then linkage as for carboxyl group Diazotized- <i>p</i> -aminobenzoic acid to introduce a carboxyl group, then linkage as for carboxyl group	Amino Amino Amino
Sugar moieties capable of forming glycosides	Reduction and diazotization of <i>p</i> -nitrophenyl glycoside	Phenol, indole, imidazole, amino
Vicinal hydroxyls (e.g., adriamycin, daunomycin)	Periodate oxidation followed by borohydride reduction of Schiff base	Amino
Ketones and aldehydes (e.g., various steroids)	Introduction of carboxyl by <i>O</i> -(carboxymethyl) hydroxylamine, then linkage as for carboxyl group	Amino
Sulphydryl	Free sulphydryl groups have been generated in active fragments of toxins by reduction and used for subsequent linkage to sulphydryl or 2-pyridyl disulfide substituents introduced into Ig or F(ab)	—

^a Many of these methods have been applied only to serum albumin or to globulin without assaying retention of drug and antibody activities in conjugates.

immunoglobulins (see Table VI) or other specific macromolecular carriers, e.g., concanavalin A.^{VI-17} These toxins possess the special advantage for antibody-mediated targeting that their cytotoxicity results from catalytic inactivation of an essential cellular constituent rather than as a result of stoichiometric combination with target molecules. In principle, this means that very few toxin molecules delivered to a target cell should be sufficient to kill that cell.

Toxins and their enzymically active A chains have been linked to antibodies by a number of different methods. Since two proteins are being linked together, one is faced with the problem of having similar reactive groups in each one. Arising from this, there is the tendency toward the formation of homo- and heteropolymers. A problem also can arise from the fact that a given amino acid residue selected for a particular linking reaction may well occur in the active center of the toxin as well as in accessible regions of the protein remote from the active site. This problem has already been alluded to in considering the immunoglobulin molecule. Another factor which must be considered is the necessity to link the toxin moiety in such a way as to allow catalysis to take place intracellularly. For example, ricin consists of two subunits connected by a disulfide bridge which must be cleaved. Early conjugation procedures linked the whole toxin to Ig such that if the binding specificity of the retained toxin B chain could be expressed, this would lead to nonspecific toxicity. As the field has developed attempts have been made to design procedures that circumvent these difficulties. For the methods of linkage of these toxins or their A chains to intact immunoglobulins or their active fragments see Table VI.

Linkage Methods

This section describes binding methods that may be applied in coupling cytotoxic agents to immunoglobulins. Table IV lists commonly used methods and the reactive groups involved.

Diazotization

Mathé and his colleagues used the diazotization reaction for linking methotrexate to a rabbit anti-mouse leukemia globulin.^{V-2} Advantages of using diazonium salts for linkage of agents to immunoglobulins include the ability to carry out the reaction in an aqueous medium at pH values between 5 and 8, the fact that no additional charge is introduced in the product, and the rapidity and completeness of the reaction at low temperature. Spacers of variable length may be introduced in the form of

TABLE IV
METHODS FOR COVALENT LINKAGE OF AGENTS TO IMMUNOGLOBULINS

Method	Reactive groups
Diazotization	Amino (aromatic), phenol, indole, imidazole, amino
Periodate oxidation-Schiff reaction	Hydroxyl, amino
Formation of an active ester intermediate (<i>N</i> -hydroxysuccinimide)	Carboxyl, amino
Activation by carbodiimide	Carboxyl, amino
Formation of a mixed-anhydride intermediate	Carboxyl, amino
Linkage by use of homobifunctional reagents	
Dimaleimides	Sulphydryl
Alkyl halides	Sulphydryl, amino, imidazole
Aryl halides	Sulphydryl, amino, phenol
Diisocyanates	Amino
Acylation agents	Amino
Diimidoesters	Amino
Dialdehydes	Amino
<p>-Benzoquinones</p>	Amino
Linkage by use of heterobifunctional reagents	
<i>N</i> -Succinimidyl-3-(2-pyridyldithio)propionate (SPDP)	Amino, sulphydryl
<i>m</i> -Maleimidobenzoyl- <i>N</i> -hydroxysuccinimide ester (MBS)	Amino, sulphydryl
Aryl azide and diazo alkyl derivatives	Various

polyphenylazo groups. Furthermore, the bonds formed by diazonium salts are easily cleaved.^{12,24} Thus this type of linkage might be preferred if dissociation of drug at target sites is desired. However, it has been found that it is difficult to control the side reactions associated with the use of diazonium compounds leading to extensive precipitation in the reaction mixture.^{1,3} Also, diazonium salts react preferentially with aromatic amino acids like tyrosine or histidine, i.e., residues that may be present in substantial numbers at or near the antigen binding site.¹² This could lead to considerable loss of antibody activity.

Periodate Oxidation

This method is applicable to linkage (a) between a carbohydrate moiety in the agent and an amino group in the immunoglobulin; (b) between an amino group in the agent and carbohydrate moieties of the immunoglobulin; or (c) via carbohydrate containing intermediaries (see Table V).

²⁴ L. A. Cohen, this series, Vol. 34, p. 102.

TABLE V
METHODS OF LINKAGE OF LOW MOLECULAR WEIGHT CYTOTOXIC AGENTS TO IMMUNOGLOBULINS^{a,b}

Drug	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
Methotrexate (MTX)	Method 1. ^{1,2} Diazotization in three steps: (1) diazotization of benzidine, (2) coupling of MTX to diazotized benzidine, and (3) coupling of IgG to the product of step 2	<p>Step 1: Benzidine reagent (720 mg of benzidine in 6.5 ml of 6 N HCl added to 31.5 ml of H₂O) and cooled to 8°. Sodium nitrite reagent, 14 ml (1.3 g of sodium nitrite in 32 ml dist. H₂O) were added slowly. The product was added to 20 ml of acetate reagent (4.5 g of Na acetate in 10 ml of dist. H₂O) with stirring</p> <p>Step 2: MTX was added to the tetrazobenzidine, dropwise with stirring. Then, 10 ml of 8 N potassium carbonate were added. The product was left at 8° for 1 hr in the dark.</p> <p>Step 3: Diazotized drug from step 2 was added to 120 ml of 16% IgG in saline plus 11.05 g of K₂CO₃ in 20 ml of water with stirring followed by 10 ml of 8 M potassium carbonate.</p>	Dialysis, against isotonic saline	<p>Drug: Molar incorporation not assayed.</p> <p>Ab: Immunodiffusion in agar. Fraction of Ab activity retained was not measured.</p> <p>MTX linked to antibody against L1210 tumor gave increased survival of tumor-bearing mice compared to mice untreated, treated with free MTX or MTX coupled to normal Ig.</p>	We and Robinson <i>et al.</i> ³ were unable to prepare active conjugates by this method owing to extensive precipitation.
	Method 2. ³⁻⁶ Water-soluble carbodiimide to link carboxyl group of MTX and amino groups of IgG forming an amide bond	<p>MTX, EDCI, and IgG were mixed in buffer. Chu and Whiteley⁴ used 40 mg of MTX, 25 mg of EDCI, and 100 mg of IgG in 0.05 M sodium bicarbonate buffer, pH 7.6 or PBS, pH 7.2. We used 20 mg of IgG, 2.4 mg of EDCI, and up to 40 mg of MTX.⁵ The reaction mixture was stirred either at room temp.⁴ or 4°.⁵</p>	Dialysis and column chromatography using BioGel P100, ⁴ BioGel P6 ⁵	<p>Drug: Molar incorporation was measured using [³H]MTX and absorbance at 370 nm. MTX activity was measured by inhibition of dihydrofolate reductase <i>in vitro</i>.⁵</p> <p>Ab: Not assayed^{3,4}; immunodiffusion in agar for antiBSA IgG⁵; membrane immunofluorescence for rabbit anti-EL4 IgG.⁵</p>	Incorporations >5-6 mol per mole of Ig led to extensive loss of Ab activity and protein precipitation.

MTX-anti-L1210 Ig conjugates increased survival of L1210 tumor-bearing mice.

MTX-anti-EL4 IgG conjugates retained Ab activity *in vitro* and specifically inhibited tumor growth *in vivo*.⁵

Drug: Incorporation measured using [³H]MTX was reported to range from 15 to 120 mol per mole depending on reaction conditions.

Ab: Immunofluorescence against ovarian tumor cells.

Conjugate prepared with rabbit Ig against ovarian carcinoma prolonged survival in tumor-bearing mice compared to those treated with free drug. Ab alone, mixture of both, or MTX coupled to nonspecific IgG.

Latif *et al.*⁸ reported loss of Ab activity when Ig was reacted with the product obtained by treating MTX with acetic anhydride. We retained Ab activity but obtained only a small amount of [³H]MTX bound to Ig when incorporation was measured after chromatography on BioGel P6. Both this material and the product of treating MTX with acetic anhydride failed to inhibit DHFR *in vitro*.

Extensive dialysis

MTX (28 mg) was suspended in 2 ml of dry acetic anhydride and heated at 100° for 30 min. Excess anhydride was evaporated in a stream of dry nitrogen at 50°. The product was redissolved immediately in 2 ml of dry dimethylformamide.

IgG (60 mg) in 10 ml at pH 8.5 was treated with this product at room temp. for 18 hr.

Method 3.⁷ MTX was treated with acetic anhydride and the product reacted with Ig.

Method 4.⁵ Preparation of an active ester of MTX followed by its reaction with amino groups in Ig producing an amide linkage.

Gel filtration on BioGel P6 or extensive dialysis.

Step 1: Preparation of the active ester. MTX (0.1 mmol) was dissolved in 1.0 ml of dry DMF. NHS (0.1 mmol) in 0.5 ml of DMF and 0.1 mmol of DCC in 0.5 ml of DMF were then added in sequence. The reaction mixture was stirred at room temp. for 1 hr and then for 18–20 hr at 4° in the dark. The yellow precipitate was discarded, and the clear orange supernatant solution containing the active ester of MTX was transferred to a capped vial and stored under nitrogen at 4° in the dark.

Drug: Same as Method 2.

Ab: Immunodiffusion in agar for anti-BSA IgG and membrane immunofluorescence using EL4 cells for anti-EL4 IgG.

Conjugates retained up to 70% antibody activity at the drug incorporation levels of 12 mol of drug per mole of IgG, but there was dramatic loss of Ab activity beyond 15 mol per mole.

MTX-anti-EL4 IgG conjugate inhibited DHFR and tumor cells *in vitro* and prolonged survival of EL4 lymphoma-bearing mice compared to drug alone. Ab alone, drug plus Ab unlinked, drug coupled to normal IgG.

(continued)

TABLE V (continued)

Drug	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
Chlorambucil (CBL)	Method 1. ^{9,10} Covalent linkage of the carboxyl group of CBL to amino groups in IgG using a water-soluble carbodiimide at low temp. to protect the alkylating bis-2-chloroethylamino group.	Step 2: Conjugation with IgG (20 mg) in 4 ml of PBS (0.01 M, pH 7.1) + 1.0 ml of DMF + 0.2 ml of active ester (added last) were stirred together at 4° for 4 hr. The mixture was centrifuged at 9500 g for 20 min, and the supernatant solution was collected.	Sephadex G-25 chromatography. CBL bound Ig appeared at void volume.	Drug: Difference spectrophotometry; Epstein reaction. ¹² Ab: Passive hemagglutination assay of ovalbumin (OA)-coated red cells for anti-OA Ab conjugates and immunofluorescence on EL4 cells for rabbit anti-EL4 IgG conjugates. Rabbit anti-EL4 IgG conjugates retained tumor inhibitory activity and tumor specificity both <i>in vitro</i> and <i>in vivo</i> .	Conjugates retained drug activity at least for 24 hr at 4°. Incorporation of >10 mol of CBL per mole of Ig interferes with Ab activity.
		Step 1: Preparation of Na salt of CBL: 12 mg of sodium methoxide was mixed with 61 mg of CBL in 2 ml of methanol. The solvent was removed <i>in vacuo</i> over sulfuric acid.			
		Step 2: 3 mg of Na CBL + 14 mg of Ig were dissolved in 1.5 ml of PBS. Then 3.5 mg of EDCI were added, and the mixture was stirred for 2 hr.			
	Method 2. ¹¹ Noncovalent linking to Ig preventing activation of CBL (low pH and low temperature).	Step 1: CBL was dissolved in 12 N HCl (1 drop/mg CBL), then desired volume of 0.01 M PBS (pH 7.1) was added.	Ultrafiltration (Amicon Centriflo CF 50A)	Drug: Epstein reaction Ab: Immunofluorescence on EL4 cells. Goat and rabbit anti-EL4 Ig conjugates that retained drug and Ab activity were tumor inhibitory both <i>in vitro</i> and <i>in vivo</i> .	Retention of Ab activity as with Method 1. Weak binding of CBL to IgG, i.e., when dialyzed against PBS at 4°, covalent conjugates retained all alkylating activity; noncovalent conjugates retained only a fraction of initial alkylating activity.
		Step 2: CBL solution was added dropwise to the desired amount of IgG solution (5 mg/ml) at 4° with stirring for 30 min (pH kept at 2).			
	Method 3. ¹³ Formation of an ester with the COOH group of Ig at alkaline pH.	CBL and Ig in 0.1 M sodium bicarbonate were incubated for 4 hr at 37°.	None	Drug: Epstein reaction; 2% incorporation of active drug in conjugate. Ab: Fraction of Ab activity retained was not determined. Conjugates made with antitheta Ig showed specific cytotoxicity toward theta-positive cells.	Polymerization of Ig inhibits drug incorporation. CBL formed nondialyzable aggregates and lost alkylating activity at alkaline pH. ¹⁴

Method 4. ⁸ Linkage to IgG via dextran bridge.	Conjugation of CBL and hexamethylenediamine to CNBr-activated dextran (M_r 18,400). Amino in dextran linked to IgG by glutaraldehyde (details not given).	Precipitation with 40% saturated NH_4SO_4 followed by dialysis.	Drug: Epstein reaction. Conjugate contained 14 mol of CBL; 8 mol of dextran; 1 mol of IgG. Ab: Cytotoxicity to K562 cells. Conjugate made with goat IgG against human myelosarcoma K562 cells retained Ab activity and inhibited the growth of target tumor cells <i>in vivo</i> . Drug: Not assayed. Ab: Not assayed. MEL-PGA-Ig conjugates made with horse anti-human lymphocyte Ig completely inhibited the response of lymphocytes in mixed lymphocyte culture. Equivalent amounts of drug alone, Ig alone, or MEL-PGA-Ig conjugate had no effect on response.	In a subsequent paper the authors discuss the problems of this method, i.e.: (a) difficulty in controlling the incorporation of PDM-PGA intermediate into Ig; and (b) unwanted cross-linkage and polymerization. ¹⁷
Melfalan (MEL)	Method 1. ¹⁵ Use of poly(L- α -glutamic acid) (PGA) as an intermediary. (See PDM.)	Details not given	Drug: Epstein reaction. PDM-PGA intermediate had PDM : PGA ratio of 45 : 1 (i.e., substitution of 16% of available carboxyl groups in PGA). PDM-PGA-Ig conjugate had PDM : PGA : Ig equal to 2 : 8 : 10 mg/ml. Ab: Cytotoxicity toward EL4 cells showing 60% retention of activity in the PDM-PGA-Ig conjugate. This conjugate was an active tumor inhibitor <i>in vivo</i> .	
N,N -Bis(2-chloroethyl)- p -phenylenediamine (PDM)	Method 1. ¹⁶ Use of poly(L- α -glutamic acid) (PGA) as an intermediary. Linkage of PDM to PGA by a carbodiimide and then linkage of PDM-PGA intermediate to Ig by a carbodiimide. ¹⁶	Step 1: Preparation of PDM-PGA intermediate: To a mixture of 25 ml of PGA (M_r 35,000, 10 mg/ml) and 14 ml of PDM (10 mg/ml) was added 1 g of EDCI. The conjugate was isolated by precipitation and washing at pH 4.0 in the cold. PDM-PGA was redissolved by addition of 1 N NaOH. Step 2: Conjugation of PDM-PGA to Ig by "concentration-solution" technique: PDM-PGA was concentrated by membrane ultrafiltration to 200 mg/ml; EDCI was then added to a final concentration of 40 mg/ml. After 2 min at room temp., the mixture was diluted 40-fold with PBS (pH 7.2) and added to an equal volume of Ig (5 mg/ml). Excess EDCI was quenched by Na acetate (final concentration 0.15 M).	Dialysis against cold PBS.	

(continued)

TABLE V (continued)

Drug	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
	Method 2. ¹⁷ Use of dextran as an intermediary. PDM and a primary amine incorporated into CNBr-activated dextran. PDM-dextran intermediate linked to Ig by glutaraldehyde via amino groups.	<p>Step 1: Coupling of hexamethylenediamine and PDM. CNBr (800 mg in acetonitrile) was added dropwise with rapid stirring to aqueous dextran (M_r 17,000, 1.0 g/liter, pH 11) at room temp. After 10 min, hexamethylenediamine (200 mg dissolved in 5 ml of water) was added, and the pH was lowered to 9.0 with 1 N HCl. The mixture was stirred for another 5 min. PDM (500 mg), dissolved in 10 ml ethanolic HCl and 40 ml of 60% aqueous propylene glycol (containing 1.2% w/v K_2HPO_4) was added dropwise. The pH was kept at 6.5 by addition of NaOH. After stirring for 15 min, the temperature was lowered to 4° and the solution was concentrated to 300 ml by hollow-fiber ultrafiltration (Amicon Corp., Model DC2). The concentrated PDM-dextran was dialyzed against distilled water until the dialyzate was free of UV-absorbing material.</p> <p>Step 2: Conjugation of PDM-dextran to Ig. To a mixture of equal vols of PDM-dex (30 mg/ml) and Ig (30 mg/ml in PBS) glutaraldehyde was added dropwise to give a final concentration of 100 μg/ml. After mixing for 1 hr at room temp., the conjugate was precipitated out with an equal volume of 80% saturated ammonium sulfate.</p>	40% saturated ammonium sulfate precipitation was followed by washing in 40% saturated ammonium sulfate and dialysis against PBS.	<p>Drug: Modified Epstein reaction. Molar incorporation not reported.</p> <p>AB: Fraction of activity retained was not reported.</p> <p>Rabbit anti-EL4 Ig-linked conjugate showed selective cytotoxicity toward mouse EL4 lymphoma cells.</p>	Glutaraldehyde-mediated conjugation of Ig and PDM-dextran is likely to produce unwanted inter- and intramolecular cross-linkage.

Trenimon [2,3,5-tris-(1-aziridinyl)-p-benzoquinone]	Method 1. ^{18,19} Reduction of disulfide groups in IgG with DTT followed by reaction between S and C-6 of the quinone ring of Trenimon.	One volume of 0.33 M DTT plus 10 vol of IgG (20 mg/ml) in isotonic PBS, pH 8, were incubated for 90 min at 37°. After dialysis overnight against 0.005 M phosphate buffer, pH 8, equal volumes of dialyzed, reduced IgG and 4.3 mM Trenimon in the same buffer were incubated for 60 min at 37°. Excess DTT has also been removed by Sephadex G-25, column chromatography. Conjugation has also been carried out without removing excess DTT.	Continuous flow dialysis against the same buffer for 24 hr	Drug: (A) Titration of uptake of acid by the active aziridine groups of Trenimon in the conjugate. (B) Epstein reaction showing 5 mol of Trenimon per mole of IgG. Ab: Cytotoxicity to guinea pig sarcoma (MCA-D). Conjugates made with rabbit IgG against this sarcoma retained both drug and Ab activity and showed selective cytotoxicity to target tumor cells <i>in vitro</i> .	Incorporation in our laboratory 2 mol of Trenimon per mole of goat IgG. ²⁰ Half-life of the alkylating activity of Trenimon in IgG conjugates at 4° (0.005 M phosphate buffer, pH 8) = 50 days.
Daunomycin (DNM) and adriamycin (ADM)	Method 2. ²¹ Introduction of sulfhydryl groups in IgG by DL-N-acetylhomocysteine thiolactone catalyzed by 2 pyridine aldolase. The thiolactone followed their reaction with the quinone ring of Trenimon.	2-Pyridine aldolase (200 mM), 0.125 mM IgG, and 250 mM DL-N-acetylhomocysteine thiolactone were stirred in deoxygenated buffer containing 0.1 M ammonium bicarbonate and 0.002 M EDTA at 4° for 4 hr under N ₂ . The pH was adjusted to 9.0 at 30-min intervals by addition of 5% trimethylamine solution in deoxygenated buffer. After dialysis overnight against deoxygenated buffer an equal volume of 4.3 mM Trenimon was added under N ₂ . Incubation of the sealed reaction mixture was carried out at 37° for 4 hr. Drug (40 mg in 1 ml of PBS) was incubated with a slight molar excess of 0.1 M NaIO ₄ for 1 hr at room temp. in the dark. Glycerol (1.0 M) was added to a final concentration of 0.05 M to consume excess periodate. The oxidized drug was incubated with 1 ml of Ig (20–25 mg in 0.15 M potassium phosphate buffer, pH 8) for 4 hr.	Dialysis overnight against phosphate buffer	Drug: Epstein reaction showing average incorporation of 7 mol per mole of IgG. Ab: Virus microhemagglutination and infectivity assays. No reduction in the titer of a rabbit anti-influenza IgG after conjugation with Trenimon.	This method avoids reduction of interchain disulfide bonds.
Daunomycin (DNM) and adriamycin (ADM)	Method 1. ²² Periodate oxidation of ADM and DNM to cleave the bond between C-3 and C-4 of the amino sugar (daunosamine) followed by Schiff base formation with the amino group of Ig and its stabilization by NaBH ₄ reduction.	In our laboratory ²⁴ , this method gave an incorporation ratio of 5:1 (ADM:Ig) and only 1% of added ADM bound to IgG. Simple mixing of ADM with IgG gave an incorporation ratio of 3:1. After borohydride	Gel chromatography using BioGel P-100 or Sepharose 6B	Drug: Ig and drug were estimated by absorbances at 280 nm and 495 nm, respectively, showing incorporation of 2–6 mol of drug per mole of Ig. Ab: Inactivation of BSA-T4 bacteriophage for conjugates made with rabbit anti-BSA Ig, showing approximately 55% retention of	In our laboratory ²⁴ , this method gave an incorporation ratio of 5:1 (ADM:Ig) and only 1% of added ADM bound to IgG. Simple mixing of ADM with IgG gave an incorporation ratio of 3:1. After borohydride

(continued)

TABLE V (continued)

Drug	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
		<p>sium carbonate buffer, pH 9.5) at room temp. for 1 hr. NaBH_4 was then added to a final concentration of 0.3 mg/ml and incubated for 2 hr at 37°.</p> <p>In a later study Hurwitz <i>et al.</i>²³ added mannitol to DNM to prevent precipitation of drug.</p>		<p>Ab activity. Cytotoxicity to mouse B leukemia cells for conjugates made with rabbit Ig against mouse B leukemia cells showing approximately 64% retention of Ab activity at 2 mol of drug per mole of Ig and 25% at 6 mol per mole.</p> <p>[³H]Uridine incorporation by mouse leukemia cells was inhibited by conjugates.</p>	<p>reduction, conjugated ADM lost anti-tumor action both <i>in vitro</i> and <i>in vivo</i>. IgG-linked ADM did not bind to DNA <i>in vitro</i>.</p> <p>Latif <i>et al.</i>⁸ reported that after conjugation of DNM to an anti-myeloma sarcoma Ab by this method, both drug and Ab activities were lost.</p>
	Method 2. ^{22,25,26} Glutaraldehyde-mediated cross-linkage between the NH_2 groups of drug and IgG.	<p>Hurwitz <i>et al.</i>²² added 100 μl of 0.1% glutaraldehyde to 1.0 ml of PBS containing 3 mg of IgG and 0.5 mg of drug at room temp. and incubated for 15 min.</p> <p>Lee <i>et al.</i>²⁵ added 100 μl of 0.25% glutaraldehyde dropwise over 5 min. The reaction was stopped with 50 μl of 1.0 M lysine after 10 min.</p> <p>Belles-Isles and Page²⁶ reported that polymerization of Ig was less when a mixture containing 0.2 mg of DNM, 1 mg of Ig, and 100 μl of 0.1% glutaraldehyde was incubated for 2-4 min at 37°.</p>	Same as Method 1	<p>Drug: Same as for Method 1.</p> <p>Hurwitz <i>et al.</i>²² reported incorporation of 7-10 mol per mole of Ig, but conjugate extensively aggregated. Lee <i>et al.</i>²⁵ reported 1-2 mol per mole; and Belles-Isles, 4 mol per mole.^{26,27}</p> <p>Ab: Same as for Method 1 for Hurwitz <i>et al.</i>²² showing no activity in the aggregated drug-containing fraction. Lee <i>et al.</i>²⁵ used immunofluorescence on guinea pig MC-D sarcoma for conjugates made with goat anti-guinea pig fibrin Ig showing retention of activity. Belles-Isles <i>et al.</i>^{26,27} tested conjugates made with rabbit anti-human CEA and α-fetoprotein Ig by cytotoxicity to mouse hematoma and human colon carcinoma cells. Conjugates prepared by Lee <i>et al.</i>²⁵ and Belles-Isles <i>et al.</i>^{26,27} were cytotoxic to appropriate antigen containing cells <i>in vitro</i>.</p>	

Method 3.²² Water-soluble carbodiimide-mediated linkage of amino group of drug to carboxyl of IgG forming an amide bond.

Method 4.^{28,29} Conjugation through a dextran bridge by periodate oxidation followed by reaction of generated aldehyde groups with amino groups in drug and IgG. Stabilization of Schiff base linkages of NaBH₄ reduction.

A mixture of 6.5 mg of EDCl, 14 mg of IgG, and 5 mg of drug in 0.1 M PBS was incubated at room temp. for 4 hr.

Step 1: Dextran (T10, T40, or T500) (2.5 g) was dissolved in 500 ml of 0.03 M NaIO₄ and incubated for 20 hr at room temp. in the dark. (It is claimed that this gives a ratio of 1 mol of periodate per glucosidic residue in dextran and represents 100% oxidation.) The product was dialyzed against water and lyophilized.

Step 2: Polyaldehyde dextran was reacted first with DNM for 20 hr at room temp. and then with IgG for 20 hr at 4°. Finally, reduction was carried out with NaBH₄ (in slight molar excess over the oxidized groups in dextran polyaldehyde).

Same as Method 1

Gel chromatography on BioGel P-60 or on Sephadex G-100

Drug: Same as for Method 1, showing 4 mol of drug per mole of IgG. Conjugates were not further characterized.

Drug: Same as for Method 1. ADM-dextran: 2-3 mol of drug linked to 1 mol of T10 and increased incorporation in dextran of high *M_r*. In the ternary complex up to 50 mol of drug were incorporated per mole of IgG.

Ab: Fraction of antibody activity remaining in conjugate not reported. Ternary conjugates made with goat and rabbit IgG against two mouse tumors were effective tumor suppressors *in vivo*, but not more so than DNM-dextran or DNM-dextran nonspecific IgG. Linkage of DNM to dextran-IgG reduced the *in vitro* cytotoxicity of the drug.²⁹

In our laboratory,²⁴ periodate-treated dextran T40 could incorporate up to 17 mol of ADM per mole of IgG. Compared to free ADM, these conjugates were less toxic to tumor cells *in vitro* and less lethal to mice. However, at equitoxic doses, ADM-dextran conjugates were more effective tumor inhibitors *in vivo* than free ADM. ADM-dextran anti-tumor conjugates were more effective tumor inhibitors *in vivo* than free drug or ADM-dextran or ADM-dextran nonspecific IgG. Borohydride reduction of conjugates led to loss of drug activity. None of the conjugates interacted with DNA *in vitro*. Latif *et al.*⁸ found that linkage of DNM to IgG through a dextran bridge retained more DNM activity than when linkage of DNM was direct to IgG.

Neocarzinostatin (NCS)

Method 1.³¹ Carbodiimide-mediated linkage between NCS protein and IgG.

A mixture containing 100 mg of NCS in 12.5 ml of saline, 13.4 mg of 1-hydroxybenzotriazole, and 19.1 mg of EDCl in 2.5 ml of saline was stirred for 1 min at

Chromatography on Sephadex G-200 removed unreacted NCS. IgG-bound NCS emerged in two overlapping peaks, the

Drug: Inhibition of *Sarcina lutea* showed retention of drug activity in the conjugate.

Ab: Immunofluorescence assay. The conjugate made with rabbit

(continued)

TABLE V (continued)

Drug	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
	Method 2. ³² Disulfide bridge between apoprotein of NCS and IgG. (SPDP to first incorporate 2-pyridyldisulfide groups into both proteins. Reduction of 2-pyridyldisulfide derivative of apoprotein with DTT followed by disulfide interchange to link it to IgG) NCS chromophore (active moiety) then added to protein conjugate.	room temp. Then 96.5 mg of IgG in 5 ml of saline were added and incubated for 17 hr with stirring at room temp. in darkness. Centrifugation for 30 min at 10,000 rpm served to remove aggregates. Step 1: Introduction of 2-pyridyldisulfide groups into IgG. IgG was incubated in 0.1 M PBS, pH 7.5, at 28° for 30 min with a 15X molar excess of SPDP. After dialysis and Sephadex G-25 chromatography, the product was stored in PBS. The incorporation of 2-pyridyldisulfide groups was 4-6 mol per mole of IgG. Step 2: Introduction of 2-pyridyldisulfide groups into apo-NCS. SPDP was reacted with the apoprotein as in step 1 except that dialysis was against 0.17 M acetate buffer, pH 5.0. One to 1.2 2-pyridyldisulfide groups were incorporated. Step 3: Thiolation: Incubation of the apo-NCS derivative with a large excess of DTT for 30 min at 28° produced apo-NCS-SH. Excess DTT and pyridine-2-thione were removed by dialysis.	first containing dimers and/or higher polymers of IgG.	IgG against human leukemia re-tained activity. It was a more effective tumor inhibitor <i>in vitro</i> than NCS alone or a mixture of NCS and IgG.	
			The apo-NCS-IgG conjugate was purified by chromatography on protein A-Sepharose CL-4B to remove unreacted apo-NCS monomer (and dimer) as well as pyridine-2-thione. After adding back the chromophore, final purification was carried out by gel chromatography on Sephadex G-25 and extensive dialysis against 0.1 M PBS (pH 7.5).	Drug: Incorporation of apo-NCS in IgG in step 4 (as measured by spectrophotometric determination of released pyridine-2-thione was 4-5 mol per mole of IgG. The conjugate restored with chromophore was shown to have drug activity by a T2-DNA degradation assay. Antibody activity of the mouse IgM myeloma protein was not assayed.	NCS consists of a single-chain acidic apoprotein (without drug activity) and a noncovalently bound nonprotein chromophore that exhibits drug activity by itself. Linkage of the apoprotein to IgG by this technique does not interfere with chromophore binding because only two amino groups located away from the binding site are available for reaction with the succinimide group of SPDP.

Step 4: Final conjugation: A 2× molar excess of apo-NCS-SH (relative to pyridyldisulfide in IgG) was incubated with the pyridyldisulfide derivative of IgG at pH 7, 28°, for 24 hr.

Step 5: Reassociation of the NCS chromophore with the apoprotein in the conjugate. The chromophore was mixed with the apo-NCS-IgG conjugate in 10–20× molar excess at pH 5 and incubated for 30 min at 10°.

Reaction in aqueous dioxane at pH 9.0. (Details not given.)

Vindesine
Method 1.^{31,32} Linkage of deacetylvincaleucoblastine acid hydrazide to Ig probably through the amino group of lysine.

Gel filtration. (Details not given.)

Drug: Difference spectrophotometry showing 3.7–4.6 mol of drug per mole of Ig.

Ab: Immunoperoxidase assay showed retention of anti-CEA activity after conjugation. Assay on CEA-producing human cancer line Calu-6 showed retention of binding activity.

AntiCEA Ig-vindesine conjugate showed preferential toxicity to CEA producing Calu cell line.

^a Abbreviations used: Ab, antibody; BSA, bovine serum albumin; DCC, dicyclohexylcarbodiimide; DMT, dimethylformamide; DTT, dithiothreitol; EDCI, 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide; Ig, immunoglobulin; M_r , molecular weight; NHS, N-hydroxysuccinimide; PBS, phosphate-buffered saline.

^b Superscript arabic numbers indicate references cited. See Key to references.

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The steps are (a) periodate oxidation of vicinal diols to yield aldehyde groups; (b) reaction between the aldehyde groups and free amino groups of agent or antibody; and (c) stabilization of the linkage by reduction with sodium borohydride. Although this method provides a high yield of conjugate,¹² especially when dextran intermediaries are used,^{V-23,V-24} it is difficult to control the reaction to obtain the extent of drug incorporation desired, and there is usually substantial loss of antibody activity in the conjugate.¹² Borohydride reduction of the Schiff base leads to the formation of a secondary amine that may not be easily cleaved *in vivo* and thus result in loss of activity in the conjugate.^{V-30}

In applying this method to linkage of periodate-oxidized dextran to the amino sugar of adriamycin and daunomycin, it may be that the aldehyde groups react with the amino group and its vicinal hydroxyl group to form an oxazolidine derivative, with the methyl ketone to form an aldol condensation product or by substitution in the aromatic moiety.^{V-30} The latter two reactions are likely to interfere with the activity of the daunomycin-adriamycin group of drugs. Periodate oxidation of immunoglobulin molecules may also give rise to unwanted inter- and intramolecular cross-linkages by the interaction of aldehyde groups with NH_2 groups in the immunoglobulin or by aldol condensation.¹²

Mixed Anhydride

Agents containing carboxyl groups can be linked to amino groups of proteins by a mixed-anhydride technique as long as amino or other sensitive groups are not present in the agent or can be suitably protected. This method has been widely applied in peptide synthesis²⁵ and in coupling various haptens, including steroid derivatives, to serum albumin. Mixed anhydrides with the monoesters of carbonic acids have been used in these procedures because they have the advantage that reaction with amino groups produces CO_2 and a alcohol as the only products aside from the amide-linked agent. Reaction of the carboxyl compound with isobutylchlorocarbonate in the presence of triethylamine in a solvent such as tetrahydrofuran or dioxane gives the mixed anhydride, which can be added directly to the protein in solvent-containing buffer at pH 8–9. A small amount of isobutyloxycarbonylated amino derivatives may also be formed. This method can be used with agents that lack carboxyl groups by introducing these groups in the form of hemisuccinates or *O*-carboxymethyl oximes (see Table III).

Burnstein and Knapp^{V-7} claimed to have produced active conjugates of MTX and an anti-mouse ovarian carcinoma antibody by allowing the

²⁵ J. R. Vaughan and R. L. Osato, *J. Am. Chem. Soc.* **74**, 677 (1952).

immunoglobulin to react with a product formed by heating MTX with acetic anhydride. However, we could not recover active MTX under conditions appropriate for hydrolysis of a mixed anhydride.^{V-5} We^{V-5} and Latif *et al.*^{V-8} failed to observe any antitumor activity in MTX derivative-Ig "conjugates" produced by this procedure.

Carbodiimides

Khorana²⁶ and Sheehan and Hess²⁷ demonstrated that carbodiimides could join suitably protected amino acids through peptide linkage. It was further demonstrated that this procedure can be carried out in aqueous media. If a free carboxyl group is presented in the agent or intermediary, carbodiimides can effect coupling presumably via free amino groups in the protein. Conversely, carbodiimides are assumed to activate carboxyl groups in immunoglobulins leading to reaction with free amino groups containing agents or intermediaries.¹² Although dicyclohexyl carbodiimide was first used for such linkage, a number of water-soluble carbodiimides (e.g., 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide, metho-*p*-toluenesulfonate, and others²⁸ have been used to link a variety of drugs including chlorambucil, *N,N*-bis(2-chloroethyl)-*p*-phenylenediamine (PDM), adriamycin/daunomycin, and MTX directly to immunoglobulins or via appropriate intermediaries (see Table V). These reagents have also been extensively used for the linkage of low molecular weight agents to proteins and of enzymes such as peroxidase and phosphatase to antibodies.^{12,29,30} It is to be noted that, since proteins contain both carboxyl and amino groups, this method is subject to unwanted cross-linkage and polymerization. The chemistry of carbodiimides has been extensively reviewed.³¹

Bifunctional Reagents

Bifunctional reagents can be exploited for forming bridges between the immunoglobulin amino acid side chains and agents (including toxins and enzymes) or intermediaries. Use has been made of both homobifunctional and heterobifunctional reagents where the reactive groups are different so that controlled, sequential activation of each group is possible. Table IV lists some of the commonly used bifunctional reagents and the groups that

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²⁷ J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.* **77**, 1067 (1958).

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²⁹ P. K. Nakane, J. Sri Ram, and G. B. Pierce, *J. Histochem. Cytochem.* **14**, 789 (1966).

³⁰ S. Bauminger and M. Wilchek, this series, Vol. 70, p. 151.

³¹ F. Kurzer and K. Douraghi-Zadeh, *Chem. Rev.* **67**, 107 (1967).

they link.³²⁻³⁴ A very useful group of heterobifunctional reagents includes those that have one relatively unreactive group that can be photoactivated to a highly reactive chemical species, e.g., nitrenes and carbenes. This type of bifunctional reagent may allow binding of agents to immunoglobulins and/or intermediaries in a sequential way: i.e., carrying out the first step in dark and the second one in the presence of activating light. However, the extremely high indiscriminate reactivity of these reagents makes it difficult to use them for controlled linkage of cytotoxic agents to immunoglobulins. Their main use has been in studies of protein subunit structure and molecular associations in membranes by cross-linking.^{34,35} Methods of cross-linking of proteins, the commonly used reagents, and the details of synthetic approaches have been reviewed.³²⁻³⁵

Bifunctional Maleimide Derivatives

N-substituted bismaleimide derivatives are mild agents that are specific for SH groups. Toxins or drugs that lack sulfhydryl groups can be linked to IgG by this method, since reactive sulfhydryl groups can be introduced into agents and IgG or its reactive fragments by various methods (Table IV). Dimaleimides have been used for linkage between sulfhydryl derivatives of IgG or F(ab) and a number of enzymes.^{36,37} The changes in ultraviolet absorption of maleimide derivatives provide a convenient method for monitoring the reaction. One disadvantage of these reagents is that they are relatively insoluble in water³² with the exception of *N,N'*-(oxydimethylene)dimaleimide.³⁶ Further information on these reagents can be found elsewhere.^{32,36}

Bifunctional Alkyl Halides

Many of these highly reactive compounds are in use as cancer chemotherapeutic agents. They are subject to nucleophilic attack by sulfhydryl, imidazole, and amino groups, etc. Reaction with sulfhydryl groups is favored at neutral to slightly alkaline pH, whereas at higher pH values reaction with amino groups is favored.³⁸ Chlorambucil has been used as a heterobifunctional reagent for the linkage of diphtheria toxin or its active fragments to antilymphocyte IgG and its (Fab)₂ (see Table VI). The car-

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³⁴ T. H. Ji, *Biochim. Biophys. Acta* **559**, 39 (1979).

³⁵ M. Das and C. F. Fox, *Annu. Rev. Biophys. Bioeng.* **8**, 165 (1979).

³⁶ P. D. Weston, J. A. Devries, and R. Wrigglesworth, *Biochim. Biophys. Acta* **612**, 40 (1980).

³⁷ K. Kato, H. Fukui, Y. Hamaguchi, and E. Ishikawa, *J. Immunol.* **116**, 1554 (1976).

³⁸ L. G. Israels and J. H. Linford, *Proc. Can. Cancer Res. Conf.*, 5th, p. 399 (1963).

TABLE VI
METHODS OF LINKAGE OF PROTEIN TOXINS AND ENZYMES TO IMMUNOGLOBULINS^{a,b}

Agent	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
Diphtheria toxin (DT)	Method 1. ¹ Substituted urea links produced in a two-stage reaction of amino groups with toluene 2,4-diisocyanate (TDIC).	Step 1: TDIC (0.1 ml) was reacted first with 25 mg of IgG in 5 ml at pH 7.5, 0°, with stirring for 30 min. The precipitated TDIC was removed by centrifugation. Step 2: DT was mixed with the supernatant solution, and the pH was adjusted to 9.5 to promote reaction with the remaining isocyanate group (molar ratio DT:IgG = 1.8:1). The mixture was stirred for 1 hr at 36°.	Dialysis and concentration followed by gel filtration through Sephadex G-200. The DT-IgG conjugate was reported to emerge at the void volume.	Agent: Molar incorporation not reported. Ab: A guinea pig antiserum against mumps antigens was purified by DEAE-cellulose column chromatography. Fraction of antibody activity remaining in the conjugate was not reported. Specific conjugate at 5×10^{-7} M inhibited protein synthesis by 50% in monkey kidney cells infected with virus and by 20% in uninfected cells.	The purification procedure did not completely separate unreacted DT from conjugate, presumably contributing to nonspecific toxicity observed with uninfected cells.
	Method 2. ² Glutaraldehyde to link Ig and DT via free amino groups in each.	Antiserum was stirred first with DNP-agarose to block the antigen binding site. DT was added, followed by 0.04% glutaraldehyde. Estimated molar ratio DT:Ig = 2:1. The mixture was stirred at room temp. for 1 hr.	The complex between DNP-agarose and DT-Ig conjugate was washed thoroughly with PBS containing 10% calf serum. Dissociation of bound conjugate was then carried out by repeated treatment with 0.1 M dinitrophenol at pH 8, centrifuging each time to collect conjugate containing bound dinitrophenol in the supernatant solution. Bound dinitrophenol was in turn removed by passage through Dowex-1. The final step was gel chromatography on Sephadex G-200, in which the conjugate emerged at the void volume; 5% of the protein applied to the column was in this fraction.	Agent: Molar incorporation not reported. Ab: AntiDNP serum was used directly. The fraction of activity remaining in the conjugate was not reported. The specific conjugate exhibited cytotoxicity toward DNP-coated tumor cells <i>in vivo</i> .	The antigen-binding site was protected during conjugation. The effect on Ig polymerization of carrying out the conjugation procedure on Ig-DNP agarose was not reported. The purification procedure would eliminate conjugate molecules that had lost antibody activity, i.e., no longer bound to DNP-agarose.

Method 3. ^{3,4} Glutaraldehyde-mediated linkage without protection of the antigen binding site.	DT and IgG (molar ratio = 2:4) were incubated with 0.04% glutaraldehyde in 0.1 M sodium phosphate, pH 6.8, for 1 hr at room temp. followed by dialysis to remove unreacted glutaraldehyde.	Gel filtration through Sephadex G-200 was followed by treatment with normal hamster tissues to remove antinormal tissue antibodies.	Agent: Molar incorporation not reported. Ab: The antibody was prepared from antisera against SV40 surface antigens by absorption to and then elution from TT 101 cells by 0.1 M glycine-HCl, pH 2.7. The IgG fraction was isolated by Na_2SO_4 precipitation. Measurement of inhibition of incorporation of [^{14}C]leucine into protein in TT 101 cells compared to NIL-2 cells <i>in vitro</i> indicated that the conjugate possessed a limited degree of specificity toward antigen-containing cells. Protective action <i>in vivo</i> was observed against an antigen-bearing sarcoma, and there was permanent regression <i>in vivo</i> of an antigen-bearing lymphoma.	Reaction conditions allow polymerization of individual proteins, and conjugate and products may not be adequately resolved by gel filtration on Sephadex G-200.
Method 4. ⁵ Amidine links were produced by reaction of amino groups with diethylmalonimide (DEM).	Diphtheria toxin and IgG (molar ratio = 7:1) were incubated in borate buffer, pH 9.4, while a 300 x total molar excess of DEM was added in four equal portions 30 min apart. After a further 30 min, 1 M NaH_2PO_4 was added to neutralize the reaction mixture.	Precipitation by addition of 0.6 volume of saturated $(\text{NH}_4)_2\text{SO}_4$ followed by resuspension in and dialysis against PBS, pH 7.	Agent: spectrophotometric assay indicated 0.1 mol of DT per mole of IgG. Ab: Rabbit anti-TNP IgG was isolated by affinity precipitation with a TNP-substituted protein. Anti-TNP antibodies were eluted by picrate and further purified by chromatography on DEAE-cellulose and Dowex 1. The eluted IgG fraction was dialyzed against PBS and then against 0.001 M phosphate, pH 7.6-8.0, to yield a preparation reported to be 92% pure as determined by precipitin analysis. The fraction of activity remaining in the conjugate was not reported. The specific conjugate selectively killed TNP-substituted HeLa cells, and this cytotoxicity was inhibited by free hapten.	Observed nonspecific cytotoxicity may be due to the failure of the limited purification procedure to remove all free DT from the conjugate preparation.

(continued)

TABLE VI (continued)

Agent	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
	Method 5, ^{6,7} Amide and tertiary amine linkage between IgG and DT produced by a mixed anhydride of chlorambucil (CBL).	<p>Step 1: Reaction of a mixed anhydride of chlorambucil with IgG at 4° to give a mustard derivative. CBL was reacted with butyl chloroformate in the presence of triethylamine at 4°. IgG in borate buffer at pH 9 was added to a 75× molar excess of the unpurified mixed anhydride of chlorambucil, and the mixture was stirred at 4° for 90 min. The Ig mustard derivative was purified by gel filtration.</p> <p>Step 2: Alkylation of DT by IgG mustard at 25–30°. DT (equimolar⁶ or in 2× molar excess⁷) was mixed with the IgG-mustard derivative and incubated for 30 hr at 25–30°.</p>	Centrifugation followed by gel chromatography on Sephadex G-150 ⁶ or G-200 ⁷ to partially resolve DT-Ig conjugate (1:1) from polymers Ig and unreacted DT.	<p>Agent: Incorporation monitored by ¹²⁵I-labeled DT. Fraction used for testing reported to contain DT-IgG (1:1), IgG, and IgG dimer.</p> <p>Ab: Thorpe <i>et al.</i>⁶ purified horse anti-human lymphocyte serum by (NH₄)₂SO₄ fractionation, absorption with red cell stroma, and treatment with DEAE-Sephadex at pH 7.8. Indirect immunofluorescence assay of binding to CLA4 cells showed that the conjugate fraction used for testing retained full antibody activity.</p> <p>The specific conjugate was 10³ times more effective than free DT in inhibiting protein synthesis in CLA4 human lymphoblastoid cells. Antibody alone did not inhibit, and DT conjugated to normal IgG was less toxic than free DT.</p> <p>In later studies, Ross <i>et al.</i>⁷ also isolated anti-mouse lymphocyte globulin by adsorption and elution from formalin-fixed mouse thymocytes and prepared F(ab)₂ from antihuman lymphocyte IgG. They chromatographed IgG and F(ab)₂ on Sephadex G-150 to remove aggregates prior to conjugation. Fluorescence end points did not differ by more than a factor of 2 for the final conjugates.</p>	Extensive polymerization of IgG, but not toxin evident from Sephadex elution pattern.

Specific conjugates inhibited protein synthesis in antigen-containing cells. For example, using Daudi cells, which are relatively insensitive to DT itself, 50% inhibition occurred at 2.4×10^{-15} M with the anti-human lymphocyte globulin conjugate, whereas the toxin itself produced no inhibition at 6.1×10^{-8} M. DT linked to normal horse IgG was without effect on these cells. Conjugates produced with F(ab')₂ were substantially as active as those produced with the parent IgG.

Diphtheria toxin, fragment A [A(DT)]

Method 1.⁸ Disulfide bridge between A(DT) and IgG formed by reaction of S-sulfonated A(DT) chain with F(ab')₂-SH.

Toxin nicked by trypsin was treated with 0.17 M Na₂SO₃ and 0.042 M Na₂S₄O₆ in the presence of 6 M urea at 37° to produce A(DT) SSO₃⁻. F(ab')₂ produced by trypsin digestion of IgG was reduced using 2 mM 2-mercaptoethanol in the presence of 2 mM EDTA at pH 7.9. F(ab')₂-SH was purified by dialysis against an acetate buffer of pH 5.6. A(DT)-SSO₃⁻ and F(ab')₂-SH were mixed in a molar ratio of 1:1 at pH 5.6. The mixture was dialyzed for 72 hr at 4° against 0.05 M glycine buffer pH 9.2, containing 2 mM EDTA to eliminate SO₃⁻.

Method 2.^{9,10} Disulfide bridge between A(DT) and IgG produced by first incorporating cystamine into IgG using a water-soluble carbodiimide and then linking A(DT)-SH by disulfide interchange (cystamine coupling).

Step 1: Incorporation of cystamine into IgG. IgG (10 mg) and cystamine 2.3×10^{-4} mol) were mixed and adjusted to pH 4.7 with HCl. Coupling was carried out with EDCI for 30 min. This was followed by addition of 1.0 M Na acetate and then dialysis

Gel chromatography through Sephadex G-150 to remove F(ab')₂ dimer, unconjugated F(ab')₂, A(DT) dimer, and A(DT).

The conjugate-containing peak on Sephadex G-150 gave one band on SDS-polyacrylamide gel electrophoresis and two bands after reduction, which corresponded in mobility to A(DT) and F(ab')₂. Unwanted dimer side products constituted less than 25% of the eluted material.⁶

Agent: ADP-ribosylation activity *in vitro* was shown to be present in the conjugate.

Ab: The antibody was a rabbit IgG against mouse L1210 leukemia cells purified by DEAE-cellulose column chromatography. The fraction of antibody activity retained in the conjugate was not reported.

The specific conjugate inhibited growth of L1210 cells more than 90% at 0.56 µg/ml (based on number of viable cells remaining at 48 hr). DT, A(DT), F(ab')₂, and a conjugate prepared with anti-DNP antibody had little effect on cell growth *in vitro*.

Agent: ¹²⁵I-labeled A(DT) incorporation and SDS polyacrylamide gel electrophoresis showed that the purified conjugate contained 1.8 mol of A(DT) per mole of IgG.

Ab: Rabbit anti-Con A was purified by affinity chromatography. Ag-

(continued)

TABLE VI (continued)

Agent	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
		<p>against 0.01 M 4-(hydroxethyl)-1-piperazineethanesulfonic acid buffer, pH 7.8, containing gentamicin and phenylmethylsulfonyl fluoride. Depending on the carboxymide added, 2.4–8.2 mol of cystamine were incorporated per mole of IgG.</p> <p>Step 2: Disulfide interchange. In a large-scale experiment A(DT) reduced by dithiothreitol and transferred into the above buffer by gel filtration, was mixed with 2.6×10^{-6} M cystaminyI IgG containing 7.2 mol of cystamine per mole of IgG (molar ratio of A(DT)-SH to IgG = 6).</p>		<p>glutination of succinyl-Con A-coated human erythrocytes showed that conjugates retained full antibody activity.</p> <p>The specific conjugate exhibited cytotoxicity toward 3T3 cells with surface-bound Con A, whereas a conjugate prepared with normal rabbit IgG was not toxic.</p>	
	<p>Method 3.^{11,12} Disulfide bridge between A(DT) and IgG. SPDP to incorporate 2-pyridyldisulfide groups into IgG followed by disulfide interchange to link A(DT)-SH.</p>	<p>Step 1: Incorporation of 2-pyridyldisulfide groups into IgG. IgG (14 mg) was stirred with a 40× molar excess of SPDP for 30 min at pH 7.4. After dialysis the derivative was found to contain an average of 4.3 2-pyridyldisulfide groups per IgG.</p> <p>Step 2: The derivative prepared in step 1 was reacted at pH 7.4 with a 3× molar excess of A(DT)-SH. The A(DT)-SH was produced from DT by tryptic cleavage and reduction at pH 7 with 0.09 M dithiothreitol followed by desalting on Sephadex G-25.</p> <p>Full details of the procedure used by Trowbridge and Domingo¹²</p>	<p>Gel chromatography on Sephacryl S-200 to remove unconjugated A(DT)-SH and A(DT)-S-S-A(DT).</p>	<p>Agent: The conjugate column peak contained multiple molecular species resolved by gel electrophoresis into IgG containing 0, 1, 2, etc. mol of A(DT) per mole of IgG.¹¹ Trowbridge and Domingo¹² reported an average of 1–2 mol of A(DT) per mole of IgG. Gilliland <i>et al.</i> reported their conjugates to contain ADP-ribosylation activity, but quantitative data were not given.</p> <p>Ab: Gilliland <i>et al.</i>¹¹ used a monoclonal mouse hybridoma-derived IgG against human colorectal carcinoma cells determined with ¹²⁵I-labeled anti-mouse F(ab')₂ antibody showed</p>	

Gelolin

Method 1.¹³ Disulfide bridge between gelolin and IgG. SPDP to incorporate 2-pyridyldisulfide groups into both proteins, then reduction of the gelolin derivative followed by disulfide interchange to link it to IgG.

were not given. They used a 6× molar excess of SPDP in step 1 to introduce an average of three 2-pyridyldisulfide groups into IgG, which was then reacted in step 2 with a 3× molar excess of A(DT)-SH for 36 hr at 4°. Their A(DT)-SH was produced from DT by tryptic cleavage by 2-mercaptoethanol followed by gel chromatography on Sephacryl S-200.

Step 1: Approximately a 2× molar excess of SPDP was used to introduce an average of 1.5 2-pyridyldisulfide groups into IgG and an average of 1.4 groups into gelolin. Both derivatized proteins were purified by gel filtration through Sephadex G-25.

Step 2: The gelolin derivative was reduced with 0.04 M dithiothreitol, gel filtered through Sephadex G-25 under nitrogen, and coupled to IgG by disulfide interchange at pH 7.5. The solution containing equimolar amounts of the two proteins was concentrated 9-fold by ultrafiltration after mixing and incubated for 3 days at room temp.

Gel chromatography through Sephadex G-100 superfine gave one peak corresponding to material of $M_r > 200,000$ partially resolved from a second peak containing 1:1 conjugate and IgG characterized by low gelolin incorporation.

partial retention of activity in conjugates.¹¹

The specific conjugate inhibited protein synthesis in antigen containing cells by 50% at a protein conc. of approximately 10^{-9} M but was not toxic toward melanoma cells at 10^{-7} M. (A conjugate prepared with cystamine-modified IgG was as toxic). A(DT) and IgG were at least 100-fold less toxic toward colorectal cells.

Trowbridge and Domingo¹² used a mouse monoclonal IgG₁ against human transferrin receptor. The fraction of antibody activity retained after conjugation was not reported. Inhibition of protein synthesis in CCRF-CEM cells was greater for the conjugate than for IgG or A(DT) alone.

Agent: Gelolin incorporation in the recovered conjugate measured by ¹³¹I-labeled gelolin content was 1.14 mol per mole of IgG (first peak of $M_r > 200,000$) and 1.04 mol per mole of IgG (second peak).

2-Pyridyldithiolation of gelolin resulted in a 50% loss of inhibitory capacity toward protein synthesis in a reticulocyte lysate. The intact conjugate had no activity in this system.

Dithiothreitol restored 9% of activity in the intact conjugate.

Ab: Monoclonal IgG2a against Thy 1.1 antigen was purified from ascites fluid by gradient elution from protein A-Sepharose. The

(continued)

TABLE VI (continued)

Agent	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
Glucose oxidase	Method 1, 14-17. Amidine links produced by reaction of amino groups with diethylmalonimide (DEM).	Same as method 4 applied to DT except that the molar ratio of enzyme to IgG was 1.6:1 and an 300 x total molar excess of DEM was added in three equal portions 30 min apart.	Precipitation of conjugate by addition of 42% satd. $(\text{NH}_4)_2\text{SO}_4$ leaving monomeric and polymerized glucose oxidase in solution. The precipitate was washed with 42% satd. $(\text{NH}_4)_2\text{SO}_4$, resuspended in PBS, pH 7.4, and dialyzed against this buffer.	fraction of antibody activity remaining after conjugation was not reported. Protein synthesis in stimulated AKR spleen cells was inhibited 50% by the 1:1 specific conjugate at 4×10^{-10} M compared to unconjugated gelonin at 3×10^{-8} M. Little or no effect was observed on lymphocytes from CBA mice (Thy 1.2 positive). The specific conjugate prolonged survival of CBA mice bearing AKR-A lymphomas.	Bifunctional imidoesters incorporating a disulfide bridge have also been used for linkage to facilitate intracellular release of agent. ¹⁸
				Ab: Rabbit anti-TNP IgG was obtained as described for method 4 applied to DT. ⁵ Goat or rabbit anti-CEA IgG ¹⁶ and rabbit anti-HT-29 cell IgG ¹⁷ were isolated by $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-cellulose column chromatography. The fraction of antibody activity remaining in conjugates was not reported. Specific conjugates were cytotoxic to antigen-containing cells <i>in vitro</i> when lactoperoxidase and $\text{I}^{-14,15,17}$ or horseradish peroxidase and arspenamine ¹⁶ were present.	

Method 2.¹⁹ Linkage based on non-covalent binding between biotin and avidin. EDCI used to couple avidin to glucose oxidase and biotin to IgE such that [IgE-biotin]-[avidin-glucose oxidase] can be formed subsequently.

Step 1: A 60× molar excess of EDCI was added to 1.0 μmole of d-biotin in 0.02 ml during 7 min at 37° followed by a further 7 min of incubation. IgE (0.01 μmol in 0.2 M borate buffer, pH 8.5) was then introduced and the mixture stirred gently for 4 hr at room temp.

Step 2: Glucose oxidase (0.086 μmol) and avidin (0.24 μmol) were mixed in 0.16 ml of H₂O and then 83 μmole of EDCI were added. After 10 min the pH was adjusted to between 5 and 6 with HCl. After a further 10 min, one volume of the pH 8.5 borate buffer was added and the incubation continued for 30 min at room temp.

The biotin-IgE conjugate was purified by dialysis against pH 8.5 borate buffer. The avidin-glucose oxidase reaction mixture was dialyzed against PBS, pH 7.4, overnight and then subjected to gel chromatography on Sepharose CL-6B. Fractions between the void volume and the elution position of IgE were pooled; glucose oxidase, avidin monomer and dimer, but not necessarily glucose oxidase dimer or polymer, would be eliminated by this procedure

EDCI gave results superior to dimethylsuberimide or glutaraldehyde in conjugating avidin to glucose oxidase.

A. Biotin-IgE conjugate.
Agent: Measurement of ¹⁴C-biotin in the dialyzed product showed 7–13 moles biotin per mole IgE.
Ab: Rat monoclonal IgE (from the immunocytooma IR162) was purified from ascitic fluid by (NH₄)₂SO₄ fractionation and gel chromatography. Binding of radioactively labeled conjugate to RBL-1 cells showed little or no impairment of binding compared to unmodified IgE.

B. Avidin-glucose oxidase conjugate.

Agent: Absorption spectrophotometry at 280 and 400 nm and spectrofluorophotometric assay of enzyme activity indicated that the conjugate contained 1 mol of glucose oxidase per 10 mol of avidin. Conjugating glucose oxidase to avidin did not significantly reduce binding to biotin-IgE-coated cells (2 mol of avidin per mol of biotin-IgE).

Binding was reduced more than 90% by prior addition of free biotin.

Selective cytotoxicity toward RBL-1 cells *in vitro* was demonstrated by treating cells with biotin-IgE, and then avidin-glucose oxidase in the presence of lactoperoxidase and I⁻.

Agent: Molar incorporation of PhC was not assayed.

Ab: Mouse antimurine lymphoma Ig was purified by (NH₄)₂SO₄ fractionation. The fraction of antibody activity remaining in the conjugate was not measured.

The conjugate was specifically cytotoxic to antigen containing target cells.

Phospholipase C (PhC)

Method 1.²⁰ Glutaraldehyde to link Ig and PhC via free amino groups in each.

Ig was first dialyzed against 0.1 M phosphate buffer, pH 6.8 at 4°. Glutaraldehyde (0.02 ml of a 10% solution) was added to a mixture of 50 mg of PhC (0.5 μmol) and approximately 50 mg of Ig in 0.5 ml of buffer. The reaction mixture was stirred for 2 hr at room temp.

Dialysis against 0.15 M NaCl overnight at 4° to remove unreacted glutaraldehyde followed by precipitation with 50% saturated (NH₄)₂SO₄ solution and a second dialysis to remove (NH₄)₂SO₄.

The conjugate was not adequately characterized.

Glutaraldehyde has also been used for linkage of L-asparaginase to Con A, but the conjugate was not active *in vivo* against lymphoma-bearing mice.²¹

(continued)

TABLE VI (continued)

Agent	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
Abrin (whole toxin)	Method 2. ²² Water-soluble carbodiimide to link PhC and IgG by amide bonds.	PhC (0.13 μ mol in 1.0 ml of PBS, pH 6) was stirred overnight at 4° with a 300 \times molar excess of 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimide and then dialyzed overnight against pH 7.2 buffer. Ig (0.033 μ mol) was added to the dialyzed product, the pH was adjusted to 9.0, and the mixture was stirred for 48 hr. Further dialysis was then carried out against pH 7.2 buffer.	Chromatography on Sepharose 6B. The fractions that contained the major protein that emerged first from the column were pooled.	Agent: Molar incorporation of PhC was not measured, but hemolytic activity was retained in the pooled column fractions used for testing. Ab: Rabbit antihuman IgG was purified by affinity chromatography using human IgG coupled to CNBr-activated agarose. The fraction of antibody activity remaining in the conjugate was not reported. Enzymic activity of the specific conjugate was inhibited by complexing with human IgG.	No direct demonstration of coupling between PhC and IgG. The prolonged reaction between PhC and the carbodiimide in the absence of IgG would favor polymerization of PhC.
	Method 1. ²³ Amide and tertiary amine linkage between IgG and abrin produced by a mixed anhydride of CBL.	Same as method 5 applied to DT. ⁶	Same as method 5 applied to DT. ⁷	Agent: Incorporation monitored by absorption spectrophotometry of ¹²⁵ I-labeled abrin content and SDS-polyacrylamide gel electrophoresis. Fractions used for testing had an M_r of approximately 210,000 and contained 0.9–1.2 mol of abrin per mole of IgG. Ab: Horse anti-human lymphocyte globulin was purified as described for method 5 applied to DT. ⁶ The fraction of antibody activity retained in the conjugate was not reported. The specific conjugate inhibited protein synthesis in Daudi cells 50% at 1.4×10^{-11} M compared to 1.5×10^{-12} M for free abrin and 1.4×10^{-10} M for abrin conjugated to normal IgG. Galactose antagonized the toxicity of abrin and abrin conjugated to normal IgG, but not abrin conjugated to the specific IgG.	Related studies have shown that abrin conjugated to horse anti-mouse lymphocyte Ig was twice as effective as the corresponding normal Ig conjugate in suppressing an immune response in mice. ²⁴
Ricin (whole toxin)	Method 1. ²⁵ Thioether and amide linkage between partially reduced IgG and ricin produced by	Step 1: A 1.7-fold molar excess of MBS was reacted with ricin at pH 7.5 for 30 min. IgG was par-	High-pressure liquid chromatography on a G3000SW TKS gel per-	Agent: Molar incorporation not reported. Ab: The antibody was monoclonal	The two-step purification procedure removed unconjugated ricin in the

m-maleimidobenzoyl-*N*-hydroxy-succinimide (MBS)

tially reduced by treatment with 0.05 *M* dithiothreitol for 30 min. After removal of residual reducing agent by passage through Sephadex G-25 equilibrated with 6.7 mM NaPO₄, pH 7.4, IgGSH was mixed with the ricin derivative in a molar ratio of 1 : 18. The reaction was allowed to proceed at room temperature for 2 hr and then stopped by addition of 6.6 mM *N*-ethylmaleimide.

Method 1.²⁶ Disulfide bridge produced by reacting Ellman's blocked F(ab') with A(R)-SH.

Ricin, fragment A [A(R)]

The reaction mixture was dialyzed against PBS. A small amount of unreacted Ellman's-blocked F(ab'), but no free A(R) were stated to be present.

Agent: Production of binary conjugate was verified by Sephadex G-100 chromatography, and reactivity with anti-rabbit F(ab')₂ and anti-ricin A chain antisera, but details were not given.

Ab: The antibody was rabbit anti-human IgG purified by an affinity technique. The F(ab')-A(R) conjugate retained full antibody activity, shown by measuring binding of conjugate, Ellman's-blocked F(ab'), and F(ab')₂ to human B cells. Binding was detected by fluorescent goat anti-rabbit F(ab')₂. The conjugate was 20-fold less effective than free A(R) in inhibiting protein synthesis in a cell-free reticulate lysate system and 50-fold less effective than whole ricin in cytotoxic action against intact IgG-bearing Daudi cells. In intact cells, protein synthesis was inhibited 79% by 1.3 × 10⁻¹⁰ *M* specific conjugate and 8% by this amount of Ellman's-blocked F(ab'). Inhibition was blocked by normal human but not bovine γ-globulin.

Disulfide linkage in a conjugate similar to that linking A and B fragments in the intact toxin may facilitate intracellular release of A(R) in catalytically active form. Antibody lacking the Fc region is less antigenic and, being smaller, may reach antibody binding sites *in vivo* more readily. However, membrane transport properties of F(ab')₂ may differ from those of intact antibody.

first (gel filtration) step and unconjugated IgG in the second (affinity chromatography) step. The low ratio of MBS to ricin and the high ratio of the maleimide derivative of ricin to Ig-SH in the coupling step would avoid ricin conjugation to more than one IgG.

anti Thy 1.2 rat IgG_{2b}. The fraction of antibody activity remaining in the conjugate was not reported. The specific conjugate inhibited protein synthesis in antigen-containing cells to approximately the same extent as ricin (50% inhibition at 5 × 10⁻⁹ *M*). The toxicity of ricin was inhibited 200-fold by lactose, but the toxicity of the conjugate only 2-fold. Protein synthesis in cells lacking antigen was not inhibited by the specific conjugate in the presence of lactose.

(continued)

TABLE VI (continued)

Agent	Principle of linkage	Procedure	Purification of conjugate	Assay of conjugate	Comments
	Method 2. ²⁷ Disulfide bridge between A(R) and IgG. SPDP to incorporate 2-pyridyldisulfide groups into IgG, which are then reduced to form the free sulfhydryl derivative, IgG-SH. Finally, reaction of IgG-SH with A(R)-SH under oxidizing conditions to produce A(R)-S-S-IgG.	<p>Step 1: A 30-fold molar excess of SPDP was used to introduce an unspecified number of 2-pyridyldisulfide groups into IgG. Free-SH groups were then formed by reduction with 5 mM dithiothreitol at room temp. for 30 min.</p> <p>Step 2: The IgG-SH derivative was mixed with a 5-fold molar excess of A(R)-SH (produced by reduction with 2-mercaptoethanol) and dialyzed extensively against PBS, pH 7.0.</p>	No purification other than dialysis reported.	<p>Agent: The molar incorporation of A(R) was not reported.</p> <p>Ab: Rabbit antimuscle μ chain IgG was purified by affinity chromatography. The specific conjugate prepared with this IgG and ^{125}I-labeled A(R) bound to the extent of 50% to Sepharose-IgM and unlabeled conjugate to the extent of 30% to immunobilized MOPC-104E (μ, γ) as measured by ^{125}I-labeled goat anti-rabbit IgG. In this latter assay, unconjugated antibody bound to the extent of 50% and a conjugate prepared with an irrelevant antibody bound less than 10%.</p>	No information was provided on the extent of formation of dimers of A(R) or of antibody during conjugation.
	Method 3. ^{11,12} Disulfide bridge between A(R) and IgG. SPDP to incorporate 2-pyridyldisulfide groups into IgG followed by disulfide interchange to link A(R)-SH.	<p>Same as method 3 applied to A(DT). Gilliland <i>et al.</i>¹¹ used a 50\times molar excess of SPDP to introduce an average of 8.9 2-pyridyldisulfide groups into IgG. This derivative was then reacted at pH 7.4 with a 3.1\times molar excess of A(R)-SH.</p>	See method 3 applied to A(DT).	<p>The specific conjugate at 5 $\mu\text{g/ml}$ inhibited protein synthesis more than 80% <i>in vitro</i> in antigen-containing cells. A(R) conjugated to an unrelated antibody did not inhibit protein or DNA synthesis. Inhibition was also demonstrated with conjugates prepared from monoclonal antibody against IgD on B cells and from affinity-purified Id antibody against IgM on BCL₁ tumor cells.</p> <p>See method 3 applied to A(DT). Using monoclonal antihuman transferrin IgG, Trowbridge and Domingo¹² found that inhibition of protein synthesis in CCRF-CEM cells was greater with conjugate than with IgG or A(R) alone. No inhibition of protein synthesis was observed in BW5147 cells even though these</p>	

cells are sensitive to intact ricin.

The A(R)-antitransferrin IgG conjugate and IgG alone were both effective in inhibition of M21 melanoma or HeLa cells growing in nude mice.

Agent: Molar incorporation was 8 mol per mole of IgM. Three out of 8 A(R) chains were reported to be active in an *in vitro* protein synthesis inhibition assay.

Ab: One antibody used was a mouse hybridoma produced IgM against Thy 1.2 antigen. There was 70% retention of antibody activity measured by complement-dependent cytotoxicity.

The A(R) preparation for conjugation inhibited protein synthesis by 50% at 5×10^{-7} M compared to 2×10^{-11} M for intact ricin and, accordingly, gave a conjugate with low nonspecific toxicity toward antigen-negative cells. The specific conjugate inhibited protein synthesis in antigen-containing cells by 50% at 10^{-10} M. Unconjugated antibody mixed with free A(R) had the same effect as A(R) alone, as did a conjugate prepared with an antibody against dextran.

Conjugates prepared with monoclonal antiTNP IgG contained 1 active A chain per IgG and were specifically cytotoxic to TNP-coated WEHI-7 cells *in vitro*.²⁹

Gel chromatography on Sephadex G-200 (IgG) or Sepharose 4B (IgM) to remove unconjugated A(R).

Step 1: Incorporation of 2-pyridylsulfide groups into Ig. Ig was reacted with an excess of 3-(2-pyridylidithio) propionic acid in the presence of EDCI.

Step 2: The derivative prepared in step 1 was reacted with an excess of A(R)-SH produced from whole toxin by reductive cleavage with 2-mercaptoethanol.

Method 4.²⁸⁻³⁰ Disulfide bridge between A(R) and Ig. 3-(2-pyridylidithio)propionic acid activated by a water soluble carbodiimide to incorporate 2-pyridylidithio groups into Ig followed by disulfide interchange to link A(R)-SH.

^a Abbreviations used: Ab, antibody; DNP, dinitrophenyl; EDCI, 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide; PBS, phosphate-buffered saline; SPDP, N-succinimidyl-3-(2-pyridylidithio)propionate; TNP, trinitrophenyl.

^b Superscript arabic numbers indicate references cited. See Key to references.

^c S-Sulfonate groups may also be introduced into proteins by amidation of amino groups with methyl-5-bromovalerimidate followed by treatment with Na₂S₂O₃.³¹

TABLE VI (continued)

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31. T. M. Chang and D. M. Neville, Jr., *J. Biol. Chem.* **252**, 1505 (1977).

boxyl group of CBL was activated by forming a mixed anhydride that was allowed to react with the amino groups in Ig at 4° to yield a modified protein bearing the alkylating mustard group. Diphtheria toxin was then added, and the temperature was raised to 25–30° to allow conjugate formation by alkylation of amino groups in the toxin.^{VI-6,7} The reaction can be easily monitored by assaying the amount of acid liberated.^{32,VI-19}

Bifunctional Isocyanates

A number of bifunctional isocyanates are available providing a wide variety of bridge lengths and reactivities. Most are insoluble in buffered salines used for dissolving immunoglobulin. The general principles of their use have been outlined.^{32,39} In addition to their reaction with amines to form substituted ureas (which is the major reaction with proteins at pH >7), they also react with alcohols forming urethanes. Side reactions in aqueous solutions (e.g., hydrolysis of the second isocyanate to an amine and its subsequent linkage to another isocyanate molecule) may lead to an aggregation of proteins through hydrophobic interaction. Several of these reagents have been used to link various proteins including diphtheria toxin to immunoglobulins.^{VI-1} Two-step procedures have been followed in an attempt to preserve antibody activity and improve the yield of conjugate, but the first step with a large excess of reagent was associated with extensive inactivation of immunoglobulins.³²

Bifunctional Acylating Agents

There are a large number of bifunctional acylating agents that vary widely in size and reactivity. Two groups have been investigated for cross-linking proteins, i.e., nitrophenyl esters of dicarboxylic acids and aromatic sulfonyl chlorides. *p*-Nitrophenyl chloroformate has been successfully used in a two-step method of linkage.³² The α -amino and ϵ -amino groups react most rapidly with these reagents.

Bifunctional Imidoesters

Imidoesters are soluble in water and can react under mild conditions with amino groups with a high degree of specificity. Parker and his colleagues used diethylmalonimidate to link glucose oxidase to anti-hapten and anti-CEA antibodies for their model studies on the interaction between antibody linked glucose oxidase and lactoperoxidase for the selective conversion of nontoxic arsenicals or halides into cytotoxic agents at

³⁹ A. F. Schick and S. J. Singer, *J. Biol. Chem.* **236**, 2477 (1961).

tumor sites (see Table VI). They should also be appropriate for amino group containing agents such as adriamycin and daunomycin. One attractive feature of these reagents, e.g., diethylmalonimidate, is that even extensive interaction with lysine residues (i.e., 85% of the residues available) can be carried out without any change in the net charge or immunological reactivity or anti-DNP or anti-BSA antibodies.³² Thus, an imidoester reacts with the same functional groups as glutaraldehyde, but gives an amidine product that retains the positive charge on the lysine side chain. γ -Globulin has been covalently linked to ferritin by this method without loss of antibody activity. Linkage may be somewhat less irreversible than with glutaraldehyde, particularly under alkaline conditions. Bifunctional imidoesters of varying chain length are commercially available, permitting the drug molecule to be spaced at various distances from the antibody moiety. In addition to this, the disulfide dimer of methyl-4-mercaptobutyrimidate will permit coupling via a disulfide bridge between drug moiety and immunoglobulin, the properties of which may be quite different from either amide bonds or borohydride reduced Schiff bases.

Aliphatic Dialdehydes

A number of dialdehydes have been investigated as cross-linking agents, e.g., glyoxal, malonaldehyde, glutaraldehyde, etc. Amino and sulfhydryl groups are the primary points of attack of these reagents.³² Glutaraldehyde has been used for linking between amino groups in the agent and immunoglobulin, essentially following the method initially described by Avrameas^{40,41} and later used by Hurwitz *et al.*^{V-22} to bind daunomycin and adriamycin to IgG (see Table V). Although the basis of dialdehyde-mediated linkage is expected to be Schiff base formation between aldehyde and amino groups, the irreversibility of bonds produced by alpha-omega dialdehydes has been attributed to the formation of Michael-type adducts.⁴²

p-Benzoquinone and Its Derivatives

p-Benzoquinone can be made to react with proteins and polysaccharides in solution via only one of its two reactive sites if added in large excess. The second site can therefore be used for coupling a drug, toxin, or intermediary that contains a reactive amino group. Ternymck and Avrameas⁴³ have described in detail the use of this reagent for coupling en-

⁴⁰ S. Avrameas, *Immunochemistry* **6**, 43 (1969).

⁴¹ M. Reichlin, this series, Vol. 70, p. 159.

⁴² F. M. Richards and J. R. Knowles, *J. Mol. Biol.* **37**, 231 (1968).

⁴³ T. Ternymck and S. Avrameas, *Ann. Immunol. (Paris)* **127C**, 197 (1976).

zymes and ferritin to IgG or a Fab fragment. *p*-Benzoquinone did not interfere with the antigen binding capacity even after substitution of 30% of available amino groups in IgG.⁴³

Bisoxiranes

These bifunctional reagents possess the virtue of permitting simultaneous introduction of a spacer group in the coupling process. They will react with both hydroxyl and amino groups under alkaline conditions, and their use with proteins has been documented.⁴⁴

m-Maleimidobenzoyl- and *m*-Maleimidocaproyl-*N*-hydroxysuccinimide Esters (MBS and MCS)

These heterobifunctional reagents are designed to couple selectively via amino and sulfhydryl groups^{45,46} under mild conditions. In the first step maleimide residues are introduced into immunoglobulin molecules by the interaction of the hydroxysuccinimide moiety of MBS or MCS with amino groups. During this step intermolecular cross-linkage is avoided because of the absence of reactive sulfhydryl groups. In the second step sulfhydryl groups in the agent react with the maleimide residue to produce a thioether link. Donkey anti-sheep IgG antibody was conjugated with β -galactosidase using MBS with retention of 85% antibody activity and 80% enzyme activity.¹³ More recently, MBS has been used to produce a cytotoxic conjugate of the A fragment of ricin with a rat monoclonal IgG₂b antibody (see Table VI). MCS has been used to link peptides related to the beta subunit of human chorionic gonadotropin to macromolecular carriers.⁴⁶

N-Succinimidyl-3-(2-pyridyldithio) Propionate (SPDP)

This reagent reacts with amino groups via its *N*-hydroxysuccinimide moiety and with aliphatic thiols via its 2-pyridyldisulfide group.⁴⁷ It is very useful for introduction of sulfhydryl groups into proteins followed by protein-protein conjugation via thiol disulfide exchange (see below). Several toxins (or their A fragments) have been conjugated to IgG (or the Fab fragment) by this method. The disulfide bridge linkages can be split by reduction or thiol-disulfide exchange, thus providing conjugates potentially cleavable in the intracellular milieu. Reaction conditions for SPDP

⁴⁴ J. Porath, this series, Vol. 34, p. 13.

⁴⁵ T. Kitagawa and T. Aikawa, *J. Biochem. (Tokyo)* **79**, 233 (1976).

⁴⁶ A. C. J. Lee, J. E. Powell, G. W. Tregear, H. D. Niall, and V. C. Stevens, *Mol. Immunol.* **17**, 749 (1980).

⁴⁷ J. Carlsson, H. Drevin, and R. Axén, *Biochem. J.* **173**, 723 (1978).

are mild, side reactions are low, and the degree of substitution with protected thiol groups is controllable and can be determined easily.⁴⁷

Noncovalent Binding

In addition to stable covalent bonds, a variety of bonds of lower energy can be used for the production of conjugates.^{9,11} Such noncovalent binding can involve hydrogen bonds, electrostatic or ionic bonds, weak interactions between dipoles or induced dipoles, and hydrophobic interactions, etc.^{11,14} Formation of a noncovalent complex of adequate stability between a drug and Ig depends on the occurrence of a sterically favorable binding site on the Ig molecule arising from the appropriate juxtaposition of hydrophobic, ionic, or polar groups such that multiple interactions can occur with that particular drug.¹⁴ Several methods have been described for noncovalently binding chlorambucil to IgG without substantially affecting either alkylating or antibody activity (see Table V). Acetylsalicylic acid is another drug that binds to IgG by electrostatic and hydrophobic interactions.⁴⁸

Linkage of Drugs to Immunoglobulins via Intermediaries and Spacers

Intermediaries have been used as a means of increasing the amount of drug incorporated per immunoglobulin molecule without the loss of antibody activity and for overcoming steric hindrance with drug activity. They include polyglutamic acid, polylysine, and various polysaccharides and synthetic polymers (Table V). The general principles of linkage of intermediaries to immunoglobulins and of drugs to intermediaries are essentially the same as those for linkage of drugs directly to immunoglobulins.

Poly-L-lysine has been used as a "piggyback" carrier of methotrexate⁴⁹ and for constructing conjugates of daunomycin so that the free drug can be released in the lysosomal milieu.⁵⁰ In principle, polylysine also provides a single carboxyl group for reaction with a free amino group in the immunoglobulin. Thus a large drug load may be incorporated into an antibody using a small number of linkage sites in that antibody. On the debit side, poly-L-lysine is extremely toxic *in vivo* and the "stickiness" of the molecule makes difficult the assay of antibody in polylysine-immunoglobulin conjugates (unpublished results).

Polysaccharides such as dextrans, Ficoll, and mannans furnish hydrophilic intermediaries obtainable in narrow molecular-weight ranges. De-

⁴⁸ Y. Y. Thomas Su and B. Jirgensons, *Biochem. Pharmacol.* **27**, 1044 (1978).

⁴⁹ W. C. Shen and H. J. P. Ryser, *Proc. Natl. Acad. Sci. U. S. A.* **75**, 1872 (1978).

⁵⁰ W. C. Shen and H. J. P. Ryser, *Biochem. Biophys. Res. Commun.* **102**, 1048 (1981).

pending upon their chemical character, agents can be linked to dextrans by direct esterification of dextran hydroxyl groups or by reaction after activation of hydroxyl groups by periodate, cyanogen halides, azide, organic cyanates, or epoxy halopropyl. Periodate oxidation of dextran yields a polyaldehyde that will react with amino groups in the agent (and immunoglobulin) to form Schiff bases that can be stabilized by borohydride reduction. This has been the most widely used method of linkage to dextran (Table V).^{5,11} Reactive moieties such as amino or carboxyl groups can be substituted in dextran to make other types of drug linkage possible (see Tables III and IV). The size of the dextran is important in determining the extent of the molar incorporation of agent and the final weight of the conjugate.

The available methods for linkage of carbohydrates to proteins have been reviewed by Aplin and Wriston.⁵¹ Those that have been used for linkage of poly- or oligosaccharides include (a) activation with cyanogen bromide; (b) linkage via triazine chloride; (c) conversion of terminal sugars to aldonates by Br₂ or I₂ oxidation, then coupling by a mixed-anhydride method or by a water-soluble carbodiimide; (d) coupling of terminal COOH groups via acyl azide, *N*-hydroxysuccinimide, water-soluble carbodiimide, or mixed-anhydride methods; (e) coupling by reaction of hydroxyl groups with succinic anhydride to introduce carboxyl groups that can be activated with *N*-hydroxysuccinimide or a carbodiimide to form amides with protein amino groups.

Synthetic polymers provide a number of possibilities in design and structural variation for the production of effective drug-antibody conjugates. Zaharko *et al.* have discussed the synthesis of both low molecular weight and polymeric carriers and possible methods of their linkage to cancer chemotherapeutic agents.¹¹ Polyvinylpyrrolidone (PVP) is water soluble and nontoxic and has been investigated as such a carrier. It has been linked to proteins by partial hydrolysis to expose carboxyl groups, blocking of secondary amines by reductive methylation, and then carboxyl activation with *N*-hydroxysuccinimide and carbodiimides. The activated PVP reacts with Ig amino groups at pH 8.5.

In the "multicompartmented model" of polymer structure proposed by Ringsdorf, desired biological properties can be introduced by incorporation of appropriate functional groups.⁶ The polymer backbone can be rendered biodegradable in the lysosomal milieu, and, if necessary, "solubilizers," "spacers," and appropriate groups for coupling antibodies can be built into the polymer. However, the practical applicability of this concept has yet to be worked out, and one can visualize problems as

⁵¹ J. D. Aplin and J. C. Wriston, *CRC Crit. Rev. Biochem.* **10**, 259 (1981).

regards uniformity of the final product, reproductibility of the synthetic procedures, susceptibility to phagocytosis, and achieving transcapillary passage and access to the tumor site.

Apart from synthetic polymers, use can be made of the high-affinity binding ($K_d = 10^{-15}$) of the egg white glycoprotein, avidin (M_r 67,000) with biotin to form intermediaries for coupling. Interestingly, only the intact ureido ring of biotin is required for this strong interaction involving the four tryptophan residues of each subunit of avidin. Thus, substitutions for linkage in both the partners are possible without interfering with the affinity of binding. In addition to its mannose, glucosamine, and oligosaccharide residues, avidin provides a number of other groups suitable for linkage using active esters or thiolated derivatives, glutaraldehyde, and water-soluble carbodiimides.^{52,53} Avidin linked to ferritin and various enzymes is now commercially available. In principle, biotin can be bound to an immunoglobulin molecule through its oligosaccharide moiety in the Fc region for subsequent complexing with avidin, which has been linked to multiple drug molecules. In fact, Bayer and Wilchek have prepared a selection of biotinyl derivatives that can be covalently linked to a variety of functional groups including carboxyl, amino, thiol, imidazole, and phenol as well as sugar residues. Methods of preparation of these derivatives have been summarized⁵² and *N*-hydroxysuccinimidobiotin (NHS-biotin) as well as a wide variety of biotinylated immunoglobulins, enzymes, and lectins are now commercially available from Vector Laboratories Inc., Burlingame, California. Philpott and colleagues were able to destroy selectively rat basophilic leukemia cells *in vitro* by exposing the cells first to a biotin-IgE conjugate followed by an avidin-glucose oxidase conjugate and the cofactors lactoperoxidase and iodide.^{VI-19}

The role of spacers in the production of active drug antibody conjugates has been investigated mainly with adriamycin and daunomycin. Spacers were designed to produce conjugates that would remain stable in the blood stream but release free drug in the lysosomal milieu. Thus linkage of daunomycin to wheat germ agglutinin via a Glc-S-Et-Arg-Leu arm⁵⁴ or to serum albumin via *N*-L-leucyl-L-alanyl-L-leucyl or *N*-L-leucyl-L-alanyl-L-leucyl-L-alanyl arms⁵⁵ provided active cytotoxic conjugates. Drug activity was lost if conjugation was directly (or via dipeptides) to

⁵² E. A. Bayer and M. Wilchek, *Methods Biochem. Anal.* **26**, 1 (1980).

⁵³ K. Hofmann, S. W. Wood, C. C. Brinton, J. A. Montibeller, and F. M. Finn, *Proc. Natl. Acad. Sci. U. S. A.* **77**, 4666 (1980).

⁵⁴ M. Monsigny, C. Kieda, A. C. Roche, and F. Delmotte, *FEBS Lett.* **119**, 181 (1980).

⁵⁵ A. Trouet, M. Masquelier, R. Baurain, and D. Deprez-De Campeneere, *Proc. Natl. Acad. Sci. U. S. A.* **79**, 626 (1982).

this macromolecular carrier. The *N*-*cis*-aconityl group was also an effective spacer in producing active daunomycin poly-D-lysine conjugates.⁵⁰

Modification of Agent, Intermediary, or Immunoglobulin for the Formation of Disulfide, Amide, and Diazo Linkages

A disulfide linkage may be desirable and in some cases obligatory for obtaining biologically active conjugates. A variety of methods have been developed for introducing sulfhydryl and disulfide groups into reactants.

SH groups can be directly introduced into reactants by reaction with *S*-acetylmercaptosuccinic anhydride (SAMSA).⁵⁶ Alternatively a disulfide may be introduced that is subsequently reduced to yield sulfhydryl groups. Disulfides used for this procedure include cystamine,^{VI-9} *N*-succinimidyl-3-(2-pyridyldithiopropionate) (SPDP)⁴⁷ and 3,3'-dimethyldithiobispropionimide.^{VI-15} These disulfide groups readily exchange with available sulfhydryl. This approach has been used in several laboratories to produce chimeric toxins (see Table VI). For example, Gilliland *et al.* coupled cystamine to concanavalin A (Con A) using a carbodiimide. The Con A disulfide derivative was then mixed with diphtheria toxin A fragment bearing a free sulfhydryl group under conditions promoting disulfide exchange to provide homopolymer-free conjugate in high yield.^{VI-9} SPDP can also be used to cross-link two moieties, both lacking free SH groups, e.g., intact IgG (or Con A) with a toxin such as gelonin. One of the 2-pyridyl disulfide-substituted proteins is made to react either with 2-mercaptoethanol or dithiothreitol for reduction of the disulfide, and the conjugate can then be formed by disulfide exchange.^{VI-11,VI-12}

In another method for conjugating two proteins both lacking in reactive sulfhydryl groups (e.g., horseradish peroxidase with IgG), King and colleagues⁵⁷ introduced sulfhydryl groups into one protein by reaction with 2-iminothiolane and 4-dithiopyridyl groups into the other protein by reaction with 2-iminothiolane in the presence of dithiodipyridine. The two modified proteins were then allowed to react to yield the conjugate and 4-thiopyridone. A different approach to preventing homodimer formation was used by Chang and Neville. They introduced *S*-sulfonate groups into one protein by amidinating available amino groups with methyl-5-bromovalerimide. The product was then converted into the *S*-sulfonated protein with Na₂S₂O₃. Finally the sulfonated protein was incubated with the free sulfhydryl group containing diphtheria toxin fragment A or ricin

⁵⁶ E. S. Rector, R. J. Schwenk, K. S. Tse, and A. H. Sehon, *J. Immunol. Methods* **24**, 321 (1978).

⁵⁷ T. P. King and I. Kochomain, *J. Immunol. Methods* **28**, 201 (1979).

fragment A at 5° in the absence of oxygen. This procedure produced a high yield of desired conjugate without the formation of intramolecular cross-linkage or homopolymers.^{VI-31} Masuho *et al.* incubated the S-sulfonated A fragment of diphtheria toxin with Fab'SH to obtain a good yield of 1:1 conjugate.^{VI-8} In a variation of this method, Yamaguchi *et al.* coupled 3,3'-dimethyldithiobispropionimide to Con A, reduced the product, and blocked the resulting sulfhydryl groups with Ellman's reagent. The disulfide derivative thus formed reacted easily with the sulfhydryl groups of the A fragment of ricin to form new S-S bonds with release of 3-nitro-4-carboxythiophenol.^{VI-18}

Amide linkages potentially susceptible to intracellular proteolytic action can be formed when carboxyl groups are present in one reactant and amino groups in the other (see Table III). If carboxyl groups are not present, various methods are available for their introduction. These have been discussed by Erlanger.⁴ Examples include reaction of sulfhydryl or hydroxyl groups with bromo- or iodoacetic acid, hydroxyl or amino groups with succinic anhydride, guanidino groups with *p*-carboxyphenylglyoxal, carbonyl groups with *O*-(carboxymethyl)hydroxylamine or hydrazides, phenols or imidazoles with diazonium salts and addition to double bonds of mercaptocarboxylic acids.

Azo bridges have been used to link two proteins by modification of amino groups. Müller and Pfeleiderer⁵⁸ selectively modified the amino groups of immunoglobulin and alkaline phosphatase by reaction with 4-hydroxyl-3-nitromethyl benzimidate hydrochloride followed by reduction and diazotization of one of the proteins. During the subsequent production of an azo bridge, no intrinsic groups (with the possible exception of thiols) are involved, thus formation of homopolymers is avoided. Reactions are well controlled so that conjugates containing various ratios of ligands to immunoglobulins can be produced. In this model study, 80–100% of antibody activity and 100% of enzymic activity could be retained in an IgG–alkaline phosphatase conjugate.

Table V lists those low molecular weight chemotherapeutic agents that have been linked to immunoglobulins and outlines the method used in each case. Table VI presents this information for protein toxins and enzymes. Examples will be found of linkage that is direct, via intermediaries as well as linkage that involves special modifications to agent or immunoglobulin. α -Amanitin, not listed in the tables, has been linked to bovine serum albumin with retention of toxic activity.⁵⁹ An azo derivative of this bicyclic octapeptide was prepared that incorporated a spacer with

⁵⁸ J. Müller and G. Pfeleiderer, *J. Appl. Biochem.* **1**, 301 (1979).

⁵⁹ J. F. Preston, R. S. Hencin, and E. J. Gabbay, *Arch. Biochem. Biophys.* **209**, 63 (1981).

a terminal carboxyl group that could be used to form an amide bond with an amino group in the protein, i.e., diazotized *p*-aminobenzoyl glycylglycine was first allowed to react with α -amanitin and then the product was linked to bovine serum albumin by use of a water-soluble carbodiimide. This method should be applicable to immunoglobulins.

Preparation of Antibodies and Fragments

Preparation of antibody-linked cytotoxic agents entails production and selection of antibody (or fragments) on the basis of their specificity and affinity toward target tissues (e.g., tumors or thymocytes). For the treatment of cancer with antibody-linked cytotoxic agents, the carrier antibodies may be specific for an individual cancer, for tumors of a given histology, or for relatively nondiscriminating markers such as oncofetal antigens. To be effective for treatment with antibody-linked cytotoxic agents (i.e., immunochemotherapy), the target antigen must be accessible for binding with the conjugate. Various membrane-bound tumor-associated antigens have been serologically defined for both experimental and human tumors. Human tumor markers or tumor-associated antigens (TAA) that have the potential to be clinically useful for immunoradioisotopic imaging or immunochemotherapy have been discussed.^{60,61}

Appropriately purified conventional polyclonal antibodies as well as hybridoma-produced monoclonal antibodies can be used for linkage to cytotoxic agents. Ideally the antibody should have high specificity, high affinity, high purity, low immunogenicity, and resistance to denaturation during conjugation procedures. The high purity and homogeneity of monoclonal antibodies against tumor markers thus make them appear to be ideal for immunochemotherapy. However, to be effective, mono- or polyclonal antibodies must retain high specificity and affinity after linkage to cytotoxic agents.

The methods of production of monoclonal antibodies against cell surface antigens are beyond the scope of this treatise. Recent issues of this series provides the details on the production of monoclonal antibodies based on hybridomas constructed with mouse myeloma lines and murine immune B cells.^{62,63} However, human myeloma cell lines are now available, and methods have been developed for the production of monoclonal

⁶⁰ K. R. McIntire, *Cancer Res.* **40**, 3083 (1980).

⁶¹ P. L. Wolf and D. Reid, in "Tumor Imaging: The Radioimmunochemical Detection of Cancer" (S. W. Burchiel and B. A. Rhodes, eds.), p. 5. Masson, Paris, 1981.

⁶² G. Galfre and C. Milstein, this series, Vol. 73, p. 3.

⁶³ B. A. L. Hurn and S. M. Chantler, this series, Vol. 70, p. 104.

antibodies of a given antigenic specificity using human hybridomas.⁶⁴ Following these methodologies, monoclonal antibodies against a number of human tumor-associated antigens (TAA) have been produced, e.g., leukemias and lymphomas,⁶⁵ colorectal carcinomas and gliomas, neuroblastomas, bronchial carcinomas, melanomas and sarcomas,⁶⁶⁻⁷⁰ and carcinoembryonic antigens (CEA).⁷¹

Production of Conventional Polyclonal Antibodies against Human Tumor Markers

With chemically characterized and highly purified human tumor markers like CEA, prostatic acid phosphatase, etc., immunization of rabbits, goats, and other suitable animals can be carried out following standard procedures.^{63,72} When antigens are available only in small amounts, effective immunization can be achieved by multiple intradermal injections with small amounts of the antigen.⁷³ When well characterized and pure TAA preparations are not available, antibodies have to be produced by immunization with dissociated tumor cells from fresh surgical specimens, cells in cultures, or TAA-containing fractions, i.e., 3 M KCl extracts of tumor cells.⁷⁴ This procedure requires that the anti-TAA antibody molecules be separated from other immunoglobulins in the antiserum, particularly those elicited by normal tissue antigens contaminating the immunizing preparation. Their removal can be carried out by repeated absorptions with normal tissue preparations until specificity is established by demonstrating reactivity with immunizing tumor, but not with normal tissues from the tumor host or with histologically unrelated human tumors.

Detailed procedures for the preparation and purification of goat and rabbit antibodies against human melanoma and renal cancer and for establishment of their specificity can be found in publications from this laboratory.^{75,76} Briefly, viable tumor cells are mixed with 2 ml of Freund's com-

⁶⁴ L. Olson and H. S. Kaplan, *Proc. Natl. Acad. Sci. U. S. A.* **77**, 6841 (1980).

⁶⁵ J. Ritz and F. Schlossman, *Blood* **59**, 1 (1982).

⁶⁶ Z. Steplewski, *Transplant. Proc.* **12**, 384 (1980).

⁶⁷ R. C. Seeger, H. M. Rosenblatt, K. Imai, and S. Ferrone, *Cancer Res.* **41**, 2714 (1981).

⁶⁸ M. J. Embleton, B. Gunn, V. S. Byers, and R. W. Baldwin, *Br. J. Cancer* **43**, 582 (1981).

⁶⁹ K. Sikora and R. Wright, *Br. J. Cancer* **43**, 696 (1981).

⁷⁰ K. Sikora and J. Phillips, *Br. J. Cancer* **43**, 105 (1981).

⁷¹ G. T. Rogers, G. A. Rawlins, and K. D. Bagshawe, *Br. J. Cancer* **43**, 1 (1981).

⁷² M. W. Chase, in "Methods in Immunology and Immunochemistry" (C. A. Williams and M. W. Chase, eds.), Vol. 2, p. 209. Academic Press, New York, 1967.

⁷³ J. L. Vaitukaitis, this series, Vol. 73, p. 46.

⁷⁴ B. J. Takács and T. Staehelin *J. Immunol. Methods* **2**, 27 (1981).

⁷⁵ T. Ghose, S. T. Norvell, A. Guclu, and A. S. MacDonald, *Eur. J. Cancer* **10**, 787 (1974).

⁷⁶ T. Ghose, P. Belitsky, J. Tai, and D. T. Janigan, *J. Natl. Cancer Inst.* **63**, 301 (1979).

plete adjuvant (Difco Laboratories, Detroit, Michigan) and injected intramuscularly into adult animals (4×10^8 cells per animal). One week later, intramuscular injections are repeated without the adjuvant. Animals receive 10 subsequent injections of cells over a 5-week period. Three days after the final injection, animals are test-bled; after serial absorption as described below, the resulting sera are tested by immunofluorescence for specific antitumor activity. Animals whose sera show a titer of 1:64 or greater are bled to obtain the maximum quantity of immune serum. The serum is inactivated at 56° and repeatedly absorbed with group AB, Rh + red cells, and homogenates of pooled normal human liver, lung, spleen, and kidney. The absorptions are repeated until the serum will react only with the immunizing tumor cells, not with skin fibroblasts, peripheral blood lymphocytes, or other normal human tissues. These absorptions result in considerable loss of specific antibodies, and immunoglobulins that are not tumor specific still persist in the preparations.

For further purification of such conventional anti-tumor antibody preparations, the IgG fraction from rabbit antisera or from mouse ascites fluid is isolated by protein A-Sepharose chromatography on DEAE-sephadex.⁷⁷ The IgG fraction can then be further purified by affinity chromatography. Well characterized tumor-associated antigens such as CEA have been immobilized on insoluble matrices and used for affinity chromatography of anti-CEA IgG.⁷⁸ Preparations containing a high proportion of specific antibody molecules have been obtained by this method. When well characterized tumor-associated antigens are not available, fractions enriched in various tumor antigens (e.g., a 3 M KCl extract of tumor cells) have been coupled to Sepharose or agarose by established procedures^{78,79} for the affinity purification. Conversely, affinity absorbents prepared from extracts of normal tissues have been used to remove contaminating anti-normal tissue antibodies from polyclonal antisera. However, this treatment will not remove contaminating antibodies of unrelated specificity originating in the rabbit or goat prior to immunization.

An alternative method is based upon the use of intact tumor cells. After treatment of cells with glutaraldehyde or formaldehyde, they may be used to adsorb tumor-specific globulins, which are then eluted after washing to remove nonspecific globulins. The procedure has been described in detail by us.⁸⁰

⁷⁷ D. R. Stanworth and M. W. Turner, in "Handbook of Experimental Immunology" (D. M. Weir, ed.), p. 6. Blackwell, Oxford, 1978.

⁷⁸ F. J. Primus and D. M. Goldenberg, *Cancer Res.* **40**, 2979 (1980).

⁷⁹ J. W. Goding, *J. Immunol. Methods* **13**, 215 (1976).

⁸⁰ A. Guclu, J. Tai, and T. Ghose, *Immunol. Commun.* **4**, 229 (1975).

Production of Fab or Fab₂ Fragments

The use of immunologically active fragments is likely to improve the transcapillary passage and diffusion properties of conjugates. Removal of the Fc moiety also renders immunoglobulins less antigenic and less likely to bind to cells that have Fc receptors (e.g. phagocytes). In the case of toxins, an interesting approach has been the coupling of the active A fragment of the toxin with Fab or Fab₂ moiety of specifically directed antibodies.

Well-established procedures are available for obtaining the Fab₂ fragment from immunoglobulin by controlled pepsin digestion.^{74,78,79} We use gel filtration and chromatographic techniques for isolation of Fab₂ and elimination of Fc. For application to mouse monoclonal antibodies, the enzymic digestion methods can be modified for mouse IgG, e.g., by using a longer digestion period with pepsin, as recommended by Casey and Tokuda.⁸¹ In our laboratory, Fab₂ is obtained by digesting 50 mg of monoclonal IgG with 1.0 mg of pepsin in 0.2 M acetate buffer (pH 4.5) at 37° for 44 hr. Peptic digestion is terminated by passing the reaction mixture through Sephadex G-150 equilibrated with 0.1 M PBS. The collected Fab₂-containing fraction is chromatographed again on protein A-Sepharose to eliminate undigested IgG. Fab₂ is precipitated with ammonium sulfate, dialyzed against PBS, and stored at 4°. If the parent IgG belongs to the IgG1 subclass, which does not consistently bind to protein A, gel filtration through Sephadex G-200 is a better method of resolving Fab₂ and IgG.

Fab is obtained by digesting 50 mg of mouse monoclonal IgG with 1 mg of papain in 0.1 M Tris-HCl, pH 8, containing 0.02 M EDTA and 10 mM cysteine at 37° for 6 hr. Digestion is terminated by adding iodacetamide (30 mM). Fab is then isolated by column chromatography on protein A-Sepharose, which binds Fc and undigested IgG. Fab bearing a free SH group is obtained by reduction of Fab₂.⁸²

The purity of Fab₂ and Fab can be established by demonstrating single bands of appropriate molecular weight on sodium dodecyl sulfate polyacrylamide gel electrophoresis and lack of reactivity against goat antibody to the Fc fragment of mouse IgG on immunoelectrophoresis.^{74,79}

Purification and Characterization of Agent-Antibody Conjugates

Low molecular weight unbound drugs, reactants, and side products can be removed from the high molecular weight conjugate-containing

⁸¹ F. B. Casey and S. Tokuda, *J. Immunol.* **105**, 1294 (1970).

⁸² M. G. Mage, this series, Vol. 70, p. 142.

fraction by gel chromatography, dialysis, or ammonium sulfate fractionation. This high molecular weight fraction will still contain any unreacted immunoglobulin, and in the case of a low molecular weight drug its resolution from conjugate is difficult, so that efforts at further purification have not been made (Table V). In conjugating proteins (toxins, enzymes, etc.) of suitable size, the choice of an appropriate gel filtration medium can allow resolution of unreacted protein, unreacted immunoglobulin, and conjugate. However, this technique is unlikely to resolve satisfactorily a mixture of various incorporation ratios of IgA₂, IgA₃, Ig₂A, etc. If conjugates with multiple incorporation ratios are formed, other methods, e.g., electrophoresis, may help in resolution. Agent-linked immunoglobulin molecules can be separated from unreacted immunoglobulins by affinity chromatography using immobilized ligands with affinity for agent, e.g., antibody against toxins or low molecular weight drugs; dihydrofolate reductase, which binds MTX;⁸³ or Poropak Q, which specifically binds adriamycin and daunomycin. The use of radioimmunoassay has led to the availability of antibodies of high specificity and affinity against a wide variety of drugs. This should encourage and use of affinity techniques for purification of drug conjugates.

Conjugates thus purified will always contain both antigen-specific and irrelevant immunoglobulin molecules if synthesized with conventional antibody preparations. Antigen unreactive molecules may also arise as a result of denaturation or blocking of the binding sites of either conventional or monoclonal antibodies. Antigen-specific conjugates can be obtained by affinity techniques using immobilized antigens or whole cells bearing accessible antigens, e.g., chromatography on a column of DNP-agarose (Table VI) of anti-DNP antibody conjugated to diphtheria toxin. Thus, in principle, by application of these methods it should be possible to purify conjugates so that the final preparations contain only immunologically active immunoglobulins that incorporate the agent. The molar incorporation of agent in immunoglobulin can be determined by various standard methods, such as isotope incorporation and absorption spectrophotometry, if the agent has a suitable chromophore group.^{63,74,V-5} Even if the spectrum of the agent overlaps that of the immunoglobulin, difference spectrophotometry can allow a reasonably accurate determination of incorporation.^{V-10,19} If the functional group in the immunoglobulin used for coupling is readily estimated (e.g., free amino groups by trinitrobenzene sulfonic acid, or carboxyl groups by a carbodiimide-based method), that group can be measured before and after coupling so that the difference gives an estimate of groups occupied in coupling.

⁸³ B. T. Kaufman, this series, Vol. 34, p. 272.

In the case of proteins conjugated to immunoglobulins, determination of the molecular weight of the conjugate by disc gel electrophoresis provides an indication of the extent of incorporation. Also, the protein can be dissociated and assayed directly if the linkage is by a disulfide bridge. These measurements, however, do not necessarily indicate the amount of active or potentially active agent in the conjugate. The rationale for assay of the activity of an agent in conjugated form should be based upon its presumed mechanism of anti-tumor action. For example, we assay conjugates containing alkylating agents (e.g. chlorambucil and Trenimon) by a colorimetric procedure based on the alkylation of nitrobenzyl pyridine (Table V). The potential activity of conjugated diphtheria toxin can be determined by measuring ADP-ribosylation activity after dissociation with dithiothreitol (Table VI). Methotrexate can be determined using dihydrofolate reductase,^{V-5} either by activity measurements or direct binding, and intercalating agents such as adriamycin can be assayed for DNA binding.⁸⁴ However, lack of activity of a conjugated agent measured by such assay procedures carried out on an intact conjugate will not exclude anti-tumor action *in vivo* if active drug can be released from protein by enzymic cleavage and/or degradative action at the tumor site. Alternatively, the conjugate may have a mode of action different from that of the free drug. That is why it is necessary to carry out a comparative analysis of tumor inhibition *in vitro* and *in vivo* caused by free drugs, antibody, and conjugates. Only by systematic study of conjugated agents can guiding principles emerge for the synthesis of optimally active conjugates. Furthermore, the results obtained should be useful in elucidating the mechanism of action of conjugated agent.

Assay of Conjugates for Retention of Antibody Activity

It is useful initially to assess the effect of coupling procedures and extent of incorporation on the retention of antibody activity by coupling the agent to antibodies against well defined antigens, such as BSA or ovalbumin, that are readily amenable to quantitative analysis. Retention of antibody activity should be measured as a function of the extent that the active drug is incorporated in the conjugate. The most promising coupling method emerging from these model studies can then be applied to antibodies against cell constituents.

During studies of drug binding employing well-defined antigens, we have generally used radial immunodiffusion because of its ease and simplicity.^{V-5} A conjugate is compared with equimolar amounts of unreacted

⁸⁴ Y. J. Schneider, R. Baurain, A. Zenebergh, and A. Trouet, *Cancer Chemother. Pharmacol.* 2, 7 (1979).

immunoglobulin, and of immunoglobulin exposed to the coupling reaction conditions with the drug omitted. Under conditions where antigen-antibody reactions are not likely to produce visible precipitates, including assay of Fab, other appropriate procedures can be selected from among well-established methods, such as those involving labeled antibodies, cytotoxicity, and hemagglutination.^{63,74} The specific binding of conjugates made with antibodies against cell-surface antigens to the surface of viable or stabilized tumor cells can be monitored by membrane immunofluorescence^{75,76} or by using labeled anti-immunoglobulins or protein A.^{63,74} Either radioisotopes or indicator enzymes⁸⁵ can be used for such labeling.

Comparison of Different Methods of Linkage of Drugs to Immunoglobulins

Studies on the comparison of different linkage methods in the same immunoglobulin-ligand system are rare. Sela's group compared three different methods of linkage of daunomycin to IgG using glutaraldehyde (Michael-type adduct), periodate borohydride (secondary amine), and a water-soluble carbodiimide (amide). The periodate-borohydride method (which cleaves the bond between C-3 and C-4 of the amino sugar of daunomycin) was reported to give the greatest retention of drug and antibody activities in the conjugate.^{V-22} However, in light of more recent results, this conclusion is now open to question. Hurwitz *et al.* coupled daunomycin to an antimouse lymphoma IgG via a dextran bridge, using periodate-oxidized dextran, which was subsequently reduced "with an amount of sodium borohydride in small excess over the total amount of oxidized groups of the polyaldehyde dextran."^{V-22} This ternary conjugate was found to be a superior tumor inhibitor compared to the free drug.^{V-29} In a more recent publication^{V-30} they also reported binding daunorubicin via its keto group to soluble^{V-34} macromolecular hydrazide derivatives of carboxymethyl dextran, polyglutamate, alginic acid and carboxymethyl cellulose using several reversible as well as a nonhydrolyzable bond. The latter was achieved by condensation of 14-bromodaunorubicin with mercapto-2-hydroxypropyl dextran. Daunorubicin attached to carriers by the nonhydrolyzable bond did not show any tumor inhibitory effect *in vitro* or *in vivo*. Macromolecular hydrazides, i.e. polyglutamyl hydrazide, polyglutamyl (hydrazide-3-hydroxylpropylamide), and carboxymethyl dextran hydrazide substituted with daunorubicin were covalently linked to a goat antimouse lymphoma IgG by periodate oxidation of the vicinal diols of Ig carbohydrate residues both with and without subsequent reduction with sodium cyanoborohydride to convert the hydrolyzable hydrazone

⁸⁵ J. E. Butler, this series, Vol. 73, p. 482.

bonds into stable hydrazido groups. However, there were considerable problems involving the precipitation of the final products, and no biological testing was done.^{V-30}

In our laboratory, incubation of periodate-oxidized dextran T40 with adriamycin yielded a dextran-linked drug product that was stable to gel filtration or repeated dialysis. Further incubation of this product, (which still contained free aldehyde groups) with rabbit anti-BSA IgG produced a ternary conjugate incorporating at least 40 mol of drug per mole of IgG with retention of at least 70% of antibody activity. The ternary conjugate could be separated from unbound dextran and IgG after gel filtration through a Sepharose CL-6B column. Unbound adriamycin was removed by dialysis prior to gel filtration. Adriamycin-containing conjugates were tumor inhibitory both *in vivo* and *in vitro*.^{V-24} However, borohydride reduction of adriamycin-dextran or the ternary conjugate completely abolished the anti-tumor action of adriamycin. We also failed to observe any consistent tumor inhibition by adriamycin linked directly to immunoglobulins by the periodate borohydride method. The most recent report from Sela's group^{V-30} and the reports that daunorubicin macromolecular conjugates were cytotoxic only when they were coupled to macromolecular carriers via spacers that could release free drug in the lysosomal milieu,^{50,54,55} support the postulation that only hydrolyzable bonds can produce active conjugates of daunomycin and adriamycin.

In our laboratory we have compared three different methods (all predicted to produce amide bonds) for coupling MTX to IgG.^{V-5} In two methods, NHS and mixed anhydride-produced intermediate derivatives were used for coupling to avoid homopolymer formation. In a third method, ECDI was used for linkage. Our results showed that the NHS-mediated method was the most effective as regards retention of drug and antibody activities and recovery of conjugate. Treatment of MTX with acetic anhydride to prepare the mixed anhydride inactivated the drug.²⁰

In a study on tumor inhibition *in vivo* by chlorambucil linked to an anti-tumor IgG preparation by covalent and noncovalent bonds, superior tumor inhibition was observed with the noncovalent conjugate.^{V-10} Warzynski *et al.* has reported that conjugation of triaziquinone (Trenimon) to IgG by a thiolation procedure^{V-21} is more reproducible and reliable method than that involving dithiothreitol-induced reduction of IgG introduced by Linford *et al.*^{V-18}

Studies on chimeric toxin molecules suggest that the toxin A chain must be linked to the carrier molecule by a disulfide bond, so that the toxin moiety can be cleaved free in the cytoplasm of the target cell. However, selective toxicity has also been observed with conjugates in which linkage was effected via thioether or alkyl bonds (Table VI). It has

also been postulated that a hydrophobic sequence⁸⁶ may play a role in uptake or toxin entry. Further studies on linkage parameters for toxins are necessary.

Avrameas and Ternynck have compared several methods used for conjugation of peroxidase and β -galactosidase to IgG (or Fab') for use as immunochemical reagents. Hydrophobic cross-linking reagents, such as toluene diisocyanate and cyanuric chloride, gave low yields of conjugates that were more susceptible to nonspecific binding to tissues or deterioration on storage as a result of the introduction of hydrophobic groups. These reagents as well as diazonium salts and carbodiimides also adversely affected the activity of many enzymes, causing either immediate loss or instability in the conjugated form. Dimaleimides also gave low yields of conjugates. In the case of peroxidase, glutaraldehyde and *m*-periodate produced homogeneous conjugates of comparable activity, and *m*-periodate usually gave a high yield of conjugate.^{12,87}

⁸⁶ D. B. Cawley, H. R. Herschman, D. G. Gilliland, and R. J. Collier, *Cell* **22**, 563 (1980).

⁸⁷ D. M. Boorsma and J. G. Streefkerk, *J. Immunol. Methods* **20**, 245 (1979).

[21] Preparation and Application of Antibodies Coupled to the A Chain of Ricin

By K. A. KROLICK, J. W. UHR, and E. S. VITETTA

There have been numerous reports describing the selective cytotoxicity of a variety of normal and neoplastic cells by toxic peptides that have been covalently coupled to specific antibodies. The synthesis and applications of such antibody-toxin conjugates have been reviewed.^{1,2}

Ricin is an example of a toxin that has been used in this manner. Ricin is obtained from castor beans and is composed of two 3×10^4 dalton polypeptide chains bridged by a disulfide bond.³ One chain (the B chain) has binding specificity for galactose and is responsible for the binding of the toxin to the surface of ricin-sensitive cells. Once bound, the other peptide chain (A chain) enters the cytoplasm and catalytically and irreversibly inhibits protein synthesis. By replacing the B chain with a specific antibody, one can create a hybrid toxin with a new target specificity.

¹ *Immunological Reviews*, Vol. 62 (1981).

² S. Olsnes and A. Pihl, *Pharmac. Ther.* **15**, 355 (1982).

³ S. Olsnes and A. Pihl, *Recept. Recognition. Ser. B* 129 (1976).

L53 ANSWER 8 OF 19

MEDLINE on STN

DUPLICATE 8

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DOCUMENT NUMBER: 95284292 PubMed ID: 7766831
TITLE: Manipulation of peptide conformations by fine-tuning of the environment and/or the primary sequence.
AUTHOR: Li S C; Kim P K; Deber C M
CORPORATE SOURCE: Division of Biochemistry Research, Hospital for Sick Children, Toronto, Ontario, Canada.
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AB The widely observed phenomenon that peptides are capable of adopting multiple conformations in different environments suggests that secondary structure formation in a peptide segment is a process involving not only the peptide itself but also the surrounding solvent media. The influence of the primary sequence and the molecular environment on peptide conformations are now investigated using synthetic peptides of amino acid sequence H₂N-(Ser-Lys)₂-Ala-X-Gly-Ala-X-Gly-Trp-Ala-X-Gly-(Lys-Ser)₃-OH, where X = Ile or Val. These two **peptides**, namely 3I (X = Ile) and 3V (X = Val), are found to **lack** defined **secondary structure** in aqueous buffer. However, discrete conformational states, e.g., alpha-helices and beta-sheets, are readily generated and interconverted for both peptides when the buffer is modulated with the addition of methanol, sodium dodecyl sulfate (SDS) micelles, or phospholipid vesicles. The role of the primary sequence in affecting peptide conformations is manifested in that peptides 3I and 3V, which differ respectively in their content of beta-branched Ile or Val residues, differ in their secondary structures at monomeric concentrations in 2 mM SDS and in mixed lipid vesicles of phosphatidic acid and phosphatidylcholine. The overall results suggest that peptide segments can be conformationally flexible entities poised to react to minor modulation in either the molecular environment or the primary sequence, a circumstance that may be relevant to protein functioning and folding.

L46 ANSWER 76 OF 122 CAPLUS COPYRIGHT 2003 ACS on STN
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 DOCUMENT NUMBER: 117:903
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 peptides and analogs thereof
 INVENTOR(S): Houghten, Richard A.; Blondelle, Sylvie
 PATENT ASSIGNEE(S): Scripps Research Institute, USA
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AB Amphiphilic peptides (Markush structure given) useful for inhibiting growth of a target cell or virus are disclosed. Ac-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Lys-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Lys-Leu-NH2 had min. inhibitory concns. of 4, 4-8, 2, and 64 .mu.g/mL against Escherichia coli, Pseudomonas aeruginosa, Staphylococcus epidermidis, and S. aureus, resp.



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(54) Title: AMPHIPHILIC PEPTIDE COMPOSITIONS AND ANALOGUES THEREOF			
<p style="text-align: center;"> Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys 1 5 10 Leu Lys Lys Leu Leu Lys Lys Leu 15 </p> <p style="text-align: right;">(I)</p>			
(57) Abstract			
<p>A process for inhibiting growth of a target cell comprising administering to a host or to a target cell or virus a biologically active peptide which includes one of the following basic structures: R₁-R₁-R₂-R₁-R₁-R₂-R₂-R₁-R₁-R₂-R₂-R₁-R₂-R₂; R₂-R₁-R₁-R₂-R₂-R₁-R₂-R₂-R₁-R₁-R₂-T₂-R₁-R₁; or R₁-R₂-R₁-R₁-R₂-R₂-R₁-R₁-R₂-R₂-R₁-R₂-R₂-R₁-R₁-R₂-R₂-R₁, wherein R₁ is a hydrophobic amino acid, and R₂ is a basic hydrophilic or neutral hydrophilic amino acid. A preferred peptide is of structural formula (I). Substitution and deletion analogues of this peptide can be prepared that have increased biological activity. Such peptides can be employed as pharmaceuticals.</p>			

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DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

⁺ It is not yet known for which States of the former Soviet Union any designation of the Soviet Union has effect.

- 1 -

AMPHIPHILIC PEPTIDE COMPOSITIONS AND
ANALOGUES THEREOF

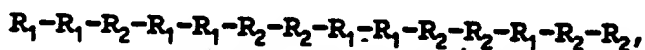
5

This invention relates to biologically active amphiphilic peptides. More particularly, this application relates to biologically active amphiphilic peptides useful in pharmaceutical compositions and to analogues of a biologically active amphiphilic peptide wherein at least one amino acid residue in the peptide has been substituted with another amino acid residue, with said analogues being commonly referred to as "substitution analogues."

15

In accordance with an aspect of the present invention, there is provided a process for inhibiting growth of a target organism such as a cell or virus which comprises administering to a host a biologically active amphiphilic peptide of the following structural formula:

20



wherein R_1 is a hydrophobic amino acid, and R_2 is a basic hydrophilic or neutral hydrophilic amino acid. The peptide is administered to the host in an amount effective to inhibit growth of a target cell or virus.

25

The hydrophobic amino acids are selected from the group consisting of Ala, Cys, Phe, Gly, Ile, Leu, Met, methionine sulfoxide, Val, Trp, and Tyr.

The basic hydrophilic amino acids are selected from the group consisting of Lys, Arg, His, orn, homoarginine (Har), 2,4-diaminobutyric acid (Dbu), and p-aminophenylalanine.

30

The neutral hydrophilic amino acids are selected from the group consisting of Asn, Gln, Ser, and Thr.

35

- 2 -

In accordance with one embodiment, R_1 is leucine. In accordance with another embodiment, R_2 is lysine.

5 In a preferred embodiment, the peptide is of the following structure (I):

I. LeuLeuLysLeuLeuLysLysLeuLeuLysLysLeuLysLys

5

10

(SEQ ID NO:1)

10 In accordance with another aspect of the present invention, there is provided a process for inhibiting growth of a target organism such as a cell or virus which comprises administering to a host a biologically active amphiphilic peptide of the following structural formula:

15 $R_2-R_1-R_1-R_2-R_2-R_1-R_2-R_2-R_1-R_1-R_2-R_2-R_1-R_1$,

wherein R_1 and R_2 are as hereinabove described. The peptide is administered to the host in an amount effective to inhibit the growth of a target cell or virus.

20 In a preferred embodiment, the peptide is of the following structure (II):

II. LysLeuLeuLysLysLeuLysLysLeuLeuLysLysLeuLeu

5

10

(SEQ ID NO:2)

25 Peptides I and II are further described in Houghten, et al., BioChromatography, Vol. 2, No. 2, pgs. 80-83 (1987).

Most preferably, Peptides I and II are acetylated with a CH_3CO -group at the N-terminus, said CH_3CO -group being hereinafter indicated by the letter X.

30 In accordance with yet another aspect of the present invention, there is provided a biologically active amphiphilic peptide of the following structural formula:

- 3 -

$R_1-R_2-R_1-R_1-R_2-R_2-R_1-R_1-R_2-R_2-R_1-R_1-R_2-R_2-R_1$, wherein R_1 is a hydrophobic amino acid, and R_2 is a basic hydrophilic or neutral hydrophilic amino acid as before defined.

5 Preferably, the peptide is acetylated with a CH_3CO -group at the N-terminal, said CH_3CO -group being represented herein by the letter X as hereinabove described.

10 Most preferably, the peptide is of the following structure (III), and acetylated at the N-terminus:

III. X-LeuLysLeuLeuLysLysLeuLeuLysLysLeuLysLys

5

10

LeuLeuLysLysLeu

15

15

(SEQ ID NO:3)

In accordance with another aspect of the present invention, there is provided a compound comprising an analogue of Peptide III, said peptide being in an amide-or carboxy-terminated (preferably amide-terminated) form. The Peptide III, also hereinafter sometimes referred to as the "parent peptide", is represented by the following structural formula, and the numbers below each amino acid residue refer to the position of the residue in the peptide.

25 LeuLysLeuLeuLysLysLeuLeuLysLysLeuLysLysLeuLeuLysLysLeu

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

(SEQ ID NO:3)

30 The parent peptide is substituted in at least one of positions 1, 3, 4 and 7-18 as follows:

Residue No.

Substituent

1

Methionine sulfoxide, Lys, or Met

3

Methionine sulfoxide, Lys, or Met

4

M thionin sulf xide, Lys, Met,

35

His, Ser, r Arg

- 4 -

- 7 Methionine sulfoxide, Lys, or Met
8 Methionine sulfoxide, Lys, or Met
9 Methionine sulfoxide
10 Methionine sulfoxide
5 11 Methionine sulfoxide, Met, Ser,
Lys, Arg, His or Gly
12 Methionine sulfoxide
13 Methionine sulfoxide, or Met
14 Methionine sulfoxide, Lys, or Met
10 15 Methionine sulfoxide, Lys, or Met
16 Methionine sulfoxide
17 Methionine sulfoxide
18 Methionine sulfoxide, or Met

15 In accordance with one embodiment, at least one of amino acid residues 1, 7, 8, 11, 14, 15, and 18 can be substituted with methionine sulfoxide.

In accordance with another embodiment, at least one of amino acid residues 1, 7, 8, 14, 15, and 18
20 can be substituted with a methionine residue.

In accordance with yet another embodiment, at least one of amino acid residues 4, 7, 8, 11, and 14 can be substituted with a lysine residue.

In accordance with a further embodiment, amino
25 acid residue 4 is substituted with a lysine residue, and amino acid residue 11 is substituted with a methionine residue.

In accordance with another embodiment, at least one of amino acid residues 4 and 11 can be
30 substituted with an arginine residue.

In accordance with yet another embodiment, at least one of amino acid residues 4 and 11 can be substituted with a histidine residue.

In accordance with another embodiment, amino
35 acid residue 11 is substituted with a glycine residue.

- 5 -

Applicants have found that when employing the substitution analogues of the parent peptide having the structural formula hereinabove described, such peptides display biological activity about equal to or greater than the parent. Such peptides are referred to as "successful substitution analogues".

As used herein, the term "substitution analogue" includes the parent peptide having the structural formula hereinabove described in which at least one amino acid residue of the peptide structure has been substituted with a different amino acid residue.

In accordance with another aspect of the present invention, there is provided a compound comprising an analogue of the parent peptide hereinabove described, said peptide being in an amide- or carboxy-terminated (preferably amide-terminated) form, wherein at least one of the amino acid residues 1 through 7, 9, 11, 12, 14, 16 or 18 is deleted from the parent peptide. In one embodiment, at least one of amino acid residues 3, 7, 11, 14 or 18 is deleted from the parent peptide. In other embodiments, amino acid residues 1 through 3, 1 through 4, 1 through 5, 1 through 6, and 1 through 7 are deleted from the peptide.

Applicants have found that when employing the deletion analogues of the parent peptide having the structural formula hereinabove described, such peptides display biological activity equal to or greater than the parent. Such peptides are referred to as "successful deletion analogues".

As used herein, the term "deletion analogue" includes the parent peptide having the structural formula hereinabove described in which at least one of the amino acid residues of the peptide structure has been deleted from the peptide.

- 6 -

In accordance with a further aspect of the present invention, there is provided a biologically active amphiphilic peptide which includes the following structural formula Y_{10} :

5 $R_1-R_1-R_2-R_2-R_1-R_2-R_2-R_2-R_1-R_2-R_2-R_1-R_1$,
wherein R_1 and R_2 are as hereinabove described.

In one embodiment, the peptide can include the following structure:

10 $Y_{10}-Z_{10}$, wherein Y_{10} is as hereinabove described, and Z_{10} is:

- (i) R_2 ;
- (ii) R_2-R_1 ; or
- (iii) $R_2-R_1-R_1$.

15 In one embodiment, the peptide has the following structural formula:

LeuLeuLysLysLeuLysLysLeuLeuLysLysLeuLeuLysLeuLeu

5

10

15

(SEQ ID NO:4)

20 The peptides hereinabove described may be acetylated with a CH_3CO -group at the N-terminal, said CH_3CO -group being indicated by the letter X as described herein.

25 The use of the hereinabove described peptides, which also includes Peptide III having substitution(s), or deletion(s) of amino acid residues, in accordance with the present invention, is effective as an antibiotic, and can be employed to inhibit, prevent or destroy the growth or proliferation of microbes, such as bacteria, fungi, viruses, or the like. Similarly,
30 such compounds can be employed as an anti-viral composition to inhibit, prevent or destroy the growth or proliferation of viruses.

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Such peptides can also be employed as a spermicide to inhibit, prevent or destroy the motility of sperm.

5 Such peptides can also be employed as anti-tumor agents to inhibit the growth of or destroy tumors, including cancer cells.

The peptides can also be employed as anti-parasitic agents to inhibit the growth of proliferation of or destroy parasites.

10 The peptides have a broad range of potent antibiotic activity against a plurality of microorganisms (target organisms), including Gram-positive and Gram-negative bacteria, fungi, protozoa, parasites and the like. Such compounds can be employed
15 for treating or controlling microbial infection caused by organisms which are sensitive to such compounds.

The peptide of the present invention can also be employed in promoting or stimulating healing of a wound in a host.

20 The term "wound healing" as used herein includes various aspects of the wound healing process. These aspects include, but are not limited to, increased contraction of the wound, increased deposition of connective tissue, as evidenced by, for example,
25 increased deposition of collagen in the wound, and increased tensile strength of the wound; i.e., the peptides increase wound breaking strength. The peptides of the present invention can also be employed so as to reverse the inhibition of wound healing caused
30 by a depressed or compromised immune system.

The compositions of the present invention can also be used in the treatment of external burns and to treat and/or prevent skin and burn infections. In particular, the compositions may be used to treat skin

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and burn infections caused by organisms such as, but not limited to, P.aeruginosa and S.aureus.

5 The peptides of the present invention can be used in the treatment of external burns and to treat and/or prevent skin and burn infections. In particular, the peptides can be used to treat skin and burn infections cause by organisms such as, but not limited to, P. aeruginosa and S. aureus.

10 The peptides are also useful in the prevention or treatment of eye infections. Such infections can be caused by bacteria such as, but not limited to, P. aeruginosa, S. aureus, and N. gonorrhoeae, by fungi such as but not limited to C. albicans and A. fumigatus, by parasites such as but not limited to A. castellani, or
15 by viruses.

 The peptides can also be effective in killing cysts, spores, or trophozoites of infection-causing organisms. Such organisms include, but are not limited to Acanthamoeba which forms trophozoites or cysts, C. albicans, which forms spores, and A. fumigatus, which
20 forms spores as well.

 The peptides can also be used as preservatives or sterilants for materials susceptible to microbial contamination. In vitro activity against bacteria is
25 exemplified hereinafter in Examples 3-9 and 11-12.

 The peptides can also be administered to plants in order to inhibit or destroy the growth or proliferation of plant pathogen target organisms such as microbes, bacteria, viruses, or parasites, fungi, cysts,
30 or spores.

 The peptides of the present invention can be administered to a target cell or host by direct or indirect application. For example, the peptide may be administ red topically r systemically.

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The peptides of the present invention can be administered to a host in particular an animal, in an effective antibiotic and/or anti-tumor and/or anti-viral and/or anti-parasitic and/or anti-microbial and/or a spermicidal amount; i.e., a growth inhibiting amount.

In general, the peptide is administered in a dosage of from about 0.1 mg to about 500 mg per kilogram of body weight, when administered systemically. When administered topically, the peptide is used in a concentration of from about 0.05 percent to about 5 percent.

The peptides, in accordance with the present invention, can be employed for treating a wide variety of hosts. In accordance with a preferred embodiment, a host can be an animal, and such animal can be a human or non-human animal.

The peptides can be employed in a wide variety of pharmaceutical compositions such as, but not limited to, those described in Remington's Pharmaceutical Sciences, 16th edition, A. Osol, ed., Mack Publishing Company, Easton, Pa. (1980), in combination with a non-toxic pharmaceutical carrier or vehicle such as a filler, non-toxic buffer, or physiological saline solution. Such pharmaceutical compositions can be used topically or systemically and can be in any suitable form such as a liquid, solid, semi-solid, injectable solutions, tablet, ointment, lotion, paste, capsule or the like. Such peptides can also be used in combination with adjuvants, protease inhibitors, or compatible drugs where such a combination is seen to be desirable or advantageous in controlling infection caused by harmful microorganisms including protozoa, viruses, parasites, and the like.

The peptides, whether substituted or unsubstituted, of the present invention, (both amide-

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and carboxy-terminated) can be synthesized by any convenient method of peptide synthesis as are well-known to skilled workers. Solid phase synthesis methods are particularly preferred.

5 Although the scope of the present invention is not intended to be limited to any theoretical reasoning, the peptides may be induced into an amphipathic α -helical configuration, which is reported to be an element responsible for the activity of known
10 antimicrobial peptides.

 The peptides described herein were prepared by the method of simultaneous multiple peptide synthesis (SMPS). This method is described in detail in Houghten, "General Method for the Rapid Solid-Phase Synthesis of
15 Large Numbers of Peptides; Specificity of Antigen-Antibody Interaction at the Level of Individual Amino Acids," Proc. Natl. Acad. Sci., U.S.A., Vol. 82, pgs. 5131-5135 (1985), in Houghten et al. "Simultaneous
20 Multiple Peptide Synthesis; The Rapid Preparation of Large Numbers of Discrete Peptides for Biological, Immunological, and Methodological Studies", Peptide Chemistry, pgs. 295-298 (1987), and in U.S. Patent No. 4,631,211, which is hereby incorporated by reference.

 The peptides can also be synthesized through
25 genetic engineering techniques. Thus, it is contemplated that within the scope of the present invention, there may be provided DNA which encodes any of the hereinabove described peptides, and it is contemplated that the peptides may be administered to a
30 host by administering DNA which encodes one of the hereinabove described peptides.

 It is also contemplated within the scope of the present invention to provide a plant which is genetically engineered with DNA which encodes one of the

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hereinabove described peptides, whereby such peptide is expressed by the plant.

For purposes of the following examples, substitution analogues of the parent peptide hereinabove described were prepared wherein various amino acid residues were substituted.

For purposes of comparison, a complete parent peptide of the structure hereinabove described can also be prepared by the SMPS method.

The invention will now be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

15 Example 1 - Peptide Synthesis

Peptide synthesis of the parent peptide (SEQ ID NO:3) hereinabove described and its substitution and deletion analogues, and of Peptide I (SEQ ID NO:1) as hereinabove described, was accomplished by using the strategy of simultaneous multiple peptide synthesis. All solvents and reagents were of analytical grade and were used without further purification. Standard N-t-Boc-protected amino acids were employed in the synthesis. The side chain functionalities used were benzyl (Ser) when serine is employed in the peptide structure, 2-Cl-Z (Lys) (Z=benzyloxycarbonyl), N¹⁸-DNP (His) when histidine is included in the peptide structure, and sulfoxide (Met). Peptide synthesis was performed beginning with 100 mg of either Boc-amino acids-Pam resin to produce C-terminal carboxyl peptide (Pam purchased from Applied Biosystems, substitution 0.56 meq/gm in an amino acyl-4-[oxymethyl]phenylacetic acid derivative of amino polystyrene) or methylbenzhydrylamine (MBHA) resin (substituted n=0.65

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meq/gm) per resin packet to produce C-terminal amide peptide.

After removing the final Boc-group and neutralizing the salt of the α -amino group, N-acetylation is carried out in the same manner as a coupling step. Instead of an amino acid, 0.3 M acetylimidazole ($C_5H_8N_2O$) in methylene chloride (CH_2Cl_2) is reacted with the free N-terminal group without adding any activator.

After synthesis, completely protected peptide resins were treated three times with 0.5 M thiophenol in DMF to remove the Nim- dinitrophenyl group from Histidine when histidine is included in the peptide structure. The final Boc-group was removed with TFA to avoid t-butylation of methionyl residues during final HF treatment when methionine is included in the peptide structure. Cleavage was performed using the Low-High HF procedure. When methionine is part of the peptide structure, one may elect not to cleave the sulfoxide group from the methionine residue(s), thus resulting in a peptide which includes methionine sulfoxide residue(s). Tam et al., J. Am. Chem. Soc. Vol. 105, p. 6442 (1983). For peptides synthesized on Pam resin, the low-HF was carried out without removing the resin from the packet, using a multiple vessel HF apparatus for two hours at 0°C. For peptides prepared using MBHA resin, the low HF procedure was performed in a common reaction vessel for two hours at 0°C. For Pam resin peptides, the low-HF mixture was evacuated from the individual reaction vessels by a water aspirator followed by a mechanical pump. The low-HF reaction vessel containing the bags with MBHA resin was emptied of the low-HF mixture by pouring off the liquid into a waste container. The bags were washed immediately with cold ether followed by alternating washes of CH_2Cl_2 ,

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DMF, CH_2Cl_2 , IPA, CH_2Cl_2 . The packets were then dried and put into individual tubes of the 24 vessel HF apparatus with 0.7 ml of anisole as scavenger. The high-HF was performed by condensing dry hydrogen fluoride at -70°C . The reaction took place at -10°C for one hour and -5°C - 0°C for the last 30 minutes. HF was evaporated using a strong flow of nitrogen. Finally, residual carbonium ion scavengers were removed by washing with dry ether.

The crude peptides were subsequently extracted with 10 percent acetic acid and subjected to RP-HPLC on an analytical reversed phase column (Vidac ODS 25 cm x 4.6 mm), using a Beckman-Altek Model 421 HPLC system and two model 110A pumps. The solvent system was composed of buffer A, 0.05 percent TFA/ H_2O , and buffer B, 0.05 percent TFA/ CH_3CN with a flow rate of 1.0 ml/min. The peptides were detected at 215 nm using a Hitachi 100-20 spectrophotometer.

Purification of the peptides was accomplished by reverse-phase HPLC on a Vidac C18 (22 mm x 25cm), 10 μm packing column with an eluting gradient composed of CH_3CN and 0.05 percent TFA. Amino acid analysis was carried out on a Beckman 6300 analyzer following hydrolysis of the peptides in constant (boiling) 6 N HCl at 110°C for 24 hours, and such analysis was within ± 10 percent of theory.

Example 2 - Antimicrobial Assays and Hemolytic Activity

For the following Examples 3-9, antimicrobial assays were carried out in 96-well tissue culture plates. Each well was incubated with a given microorganism (Escherichia coli, Staphylococcus epidermidis, or Pseudomonas aeruginosa) suspended in LB medium. Up n the additi n f the parent p ptide having the structural f rmula her inabove d scribed, or its

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substitution analogues, or of Peptide I (dissolved in 1 X PBS, pH 7.0) each well contained a final cell density of 1.0×10^6 colony forming units (CFU)/ml. The final peptide concentrations ranged from 1.5 $\mu\text{g/ml}$ to greater than 100 $\mu\text{g/ml}$.

Addition of peptides to the wells was defined as time zero. At twenty hours, the plates were placed in a Titertek Multiskan apparatus and the O.D.620 determined. The plates as well as the initial inoculum were incubated at 37°C.

Five wells per plate contained media alone, while five others contained medium plus cells. These controls were used to eliminate the possibility of media contamination while providing a measure of uninhibited growth of the microorganisms.

The degree of peptide activity was determined by comparing the substitution analogues with uninhibited growth of the control cells over a twenty-hour period. The effective growth inhibition of the substitution analogues is listed in the examples and tables below.

The hemolytic activity of the parent peptide and its substitution analogues and of Peptide I was examined with human red blood cells. Ten μl of blood were suspended in isotonic PBS buffer (pH 7) to reach a concentration of 2 percent of cells in PBS, followed by the addition of the peptide to reach a final volume of 1 ml. The peptide concentration is 100 $\mu\text{g/ml}$. The suspension was gently mixed and then incubated for 30 minutes at 37°C. The samples were centrifuged at 1000 g for five minutes. The supernatant was separated from the pellet and the optical density was measured at 414 nm. 100 Percent hemolysis was determined by disrupting the human erythrocytes in pure H₂O.

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Example 3 - Parent peptide and analogues with methionine sulfoxide substitutions.

A parent peptide having the following structural formula:

5



as hereinabove described and analogues thereof wherein a methionine sulfoxide residue was substituted for various amino acid residues of the parent peptide were prepared as hereinabove described in Example 1 and tested for minimum inhibitory concentration with respect to E. coli, P. aeruginosa, and S. epidermidis at a concentration given in $\mu\text{g/ml}$, and for percent hemolysis of human red blood cells as hereinabove described in Example 2. The minimum inhibitory concentration and percent hemolysis (at a concentration of $100 \mu\text{g/ml}$) are listed below in Table I. As used herein, the heading "Amino Acid Residue Substituted" refers to the number of the amino acid residue in the peptide which is substituted with a desired amino acid residue. All other residues remain the same as that of the normal peptide sequence. The term "Minimum Inhibitory Concentration" (MIC) as used herein indicates the minimum concentration of peptide in $\mu\text{g/ml}$ needed to achieve 100 percent effective growth inhibition of the organism.

10

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TABLE I

Amino Acid Residue Substituted	<u>E. Coli</u>	<u>P. Aeruginosa</u>	<u>S. epidermidis</u>	<u>Percent Hemolysis</u>
None (parent)	10	75	2.5	6.0
1 (SEQ ID NO:5)	5	30	10	0.4
2 (SEQ ID NO:6)	10	40	20	67.6
3 (SEQ ID NO:7)	10	30	5	3.3
4 (SEQ ID NO:8)	10	30	5	0.3
5 (SEQ ID NO:9)	10	10	10	12.2
6 (SEQ ID NO:10)	10	30	10	12.4
7 (SEQ ID NO:11)	5	5	10	3.7
8 (SEQ ID NO:12)	5	10	5	5.2
9 (SEQ ID NO:13)	10	20	10	5.4
10 (SEQ ID NO:14)	10	30	10	9.7
11 (SEQ ID NO:15)	10	5	10	0.1
12 (SEQ ID NO:16)	10	20	10	9.4
13 (SEQ ID NO:17)	10	30	10	2.4
14 (SEQ ID NO:18)	5	5	5	2.2
15 (SEQ ID NO:19)	5	10	5	4.2
16 (SEQ ID NO:20)	10	10	20	5.8
17 (SEQ ID NO:21)	10	30	20	8.7
18 (SEQ ID NO:22)	5	10	10	7.1

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Example 4 - Analogues with lysine substitutions

Analogues of the parent peptide described in Example 3 were prepared as hereinabove described in Example 1, wherein a lysine residue was substituted for various amino acid residues. The analogues were then tested for MIC with respect to E. coli, P. aeruginosa, and S. epidermidis, and for hemolytic activity as hereinabove described in Example 2. The MIC and hemolytic activity is listed below in Table II.

TABLE II

Amino Acid Residue Substituted	Min. Inhibitory Concentration (μ g/ml)			
	E. Coli	P. Aeruginosa	S. epidermidis	% Hemolysis
1 (SEQ ID NO:23)	5	30	25	4.5
3 (SEQ ID NO:24)	5	30	10	5.3
4 (SEQ ID NO:25)	2.5	30	5	4.0
7 (SEQ ID NO:26)	5	30	5	3.1
8 (SEQ ID NO:27)	5	20	10	5.5
11 (SEQ ID NO:28)	5	30	5	3.2
14 (SEQ ID NO:29)	5	20	10	6.1
15 (SEQ ID NO:30)	10	20	5	9.6
18 (SEQ ID NO:31)	10	20	10	16.8

Example 5 - Analogues with methionine substitutions

Analogues of the parent peptide described in Example 3 were prepared as described in Example 1, wherein a methionine residue was substituted for various amino acid residues. The analogues were then tested for MIC with respect to E. coli, P. aeruginosa, and S. epidermidis, and for hemolytic activity as hereinabove described in Example 2. The MIC and hemolytic activity is listed below in Table III.

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TABLE III

Amino Acid Residue Substituted	Min. Inhibitory Concentration $\mu\text{g/ml}$			
	E. Coli	P. Aeruginosa	S. epidermidis	% Hemolysis
1 (SEQ ID NO:5)	5	20	5	6.3
2 (SEQ ID NO:6)	10	40	10	15.0
3 (SEQ ID NO:7)	20	30	20	4.4
4 (SEQ ID NO:8)	10	20	10	5.3
5 (SEQ ID NO:9)	30	40	20	20.0
6 (SEQ ID NO:10)	10	30	10	26.8
7 (SEQ ID NO:11)	5	10	10	8.2
8 (SEQ ID NO:12)	5	30	10	8.1
9 (SEQ ID NO:13)	10	30	10	18.1
10 (SEQ ID NO:14)	10	30	10	39.6
11 (SEQ ID NO:15)	10	30	10	9.2
12 (SEQ ID NO:16)	10	30	20	53.2
13 (SEQ ID NO:17)	10	30	10	7.1
14 (SEQ ID NO:18)	5	30	5	9.7
15 (SEQ ID NO:19)	5	30	5	8.4
16 (SEQ ID NO:20)	10	50	10	13.7
17 (SEQ ID NO:21)	20	30	20	13.9
18 (SEQ ID NO:22)	5	20	10	5.5

Example 6 - Analogues with substitutions at amino acid residues 4 and 11

Analogues of the parent peptide of Example 3 were prepared as described in Example 1, wherein amino acid residues 4 and 11 were substituted with lysine, arginine, histidine, serine, methionine or methionine sulfoxide residues. The structures of these analogues were as follows:

Analyse No.	Structure
1	X(SEQ ID NO:32)-NH ₂
2	X(SEQ ID NO:33)-NH ₂
3	X(SEQ ID NO:34)-NH ₂
4	X(SEQ ID NO:35)-NH ₂
5	X(SEQ ID NO:36)-NH ₂
6	X(SEQ ID NO:37)-NH ₂
7	X(SEQ ID NO:38)-NH ₂
8	X(SEQ ID NO:39)-NH ₂

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9
10X(SEQ ID NO:40)-NH₂
X(SEQ ID NO:41)-NH₂

5 The analogues were then tested for MIC with respect to E. coli, P. aeruginosa, and S. epidermidis, and for hemolytic activity as described in Example 2. The results for each analogue are given in Table IV below:

10 **TABLE IV**

Min. Inhibitory Concentration (ug/ml)

Analogue No.	<u>E. Coli</u>	<u>P. Aeruginosa</u>	<u>S. epidermidis</u>	<u>% Hemolysis</u>
15 1	>100	50	100	0.0
2	>100	>100	25	0.0
3	25	25	25	0.1
4	25	25	10	0.0
20 5	10	10	5	3.2
6	100	100	100	N/A
7	10	25	5	0.0
8	5	5	5	0.9
9	100	50	>100	N/A
25 10	100	100	100	N/A

Example 7 - Analogues with arginine or histidine substitutions

30 Analogues of the parent peptide described in Example 3 were prepared according to Example 1 wherein arginine or histidine substitutions were made for amino acid residues 4 or 11. The analogues which were prepared are as follows:

35

Analogue No.	Amino Acid Residue Substituted	Substituent
40 1 (SEQ ID NO:42)	4	Arginine
2 (SEQ ID NO:43)	4	Histidine
3 (SEQ ID NO:44)	11	Arginin
4 (SEQ ID NO:45)	11	Histidine

- 20 -

The analogues were then tested for MIC with respect to E. coli, P. aeruginosa, and S. epidermidis, and for hemolytic activity as described in Example 2. The results are given in Table V below.

5

TABLE V

		<u>Min. Inhibitory Concentration (μg/ml)</u>			
10	<u>Analogue No.</u>	<u>E.</u>	<u>P.</u>	<u>S.</u>	<u>%</u>
		<u>Coli</u>	<u>Aeruginosa</u>	<u>epidermidis</u>	<u>Hemolysis</u>
	1	5	5	2.5	3.0
	2	5	10	2.5	2.1
15	3	10	5	2.5	0.3
	4	5	1.5	1.5	2.5

Example 8

20 Analogues of the parent peptide of Example 3 were prepared as described in Example 1, wherein either amino acid residue 4, 7, or 11 was substituted with a methionine sulfoxide residue. The substitution analogues were then processed further to produce

25 fractions of each substitution analogue containing varying proportions of L-methionine-L-sulfoxide residues and L-methionine-D-sulfoxide residues. For purposes of explanation, the terms under the "Proportion" column in Table VI indicate that a certain percentage of the

30 peptide included an L-methionine-L-sulfoxide residue or an L-methionine-D-sulfoxide residue, and an enumerated percentage of the peptides included the other of the L-methionine-L-sulfoxide and L-methionine-D-sulfoxide residue. It has not been determined which percentage

35 corresponds to the L-methionine-L-sulfoxide residue and which percentage corresponds to the L-methionine-D-sulfoxide residue. The analogue preparations were then tested for MIC with respect to E. coli, P. aeruginosa, and

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S.epidermidis, and for hemolytic activity as described in Example 2. The results are given in Table VI below.

TABLE VI

Amino Acid Residue	Pro- portion	E. Coli	Min. Inhibitory Concentration ($\mu\text{g/ml}$)			% Hemo- lysis
			<u>P.Aeru- ginosa</u>	<u>S.epi- demidia</u>		
4 (SEQ ID NO:8)	50/50	10	5	5		0.0
4 (SEQ ID NO:8)	60/40	>10	5	5		0.1
4 (SEQ ID NO:8)	0/100	>10	5	5		0.5
4 (SEQ ID NO:8)	100/0	>10	>5	>10		1.2
7 (SEQ ID NO:11)	70/30	10	>5	>10		0.9
7 (SEQ ID NO:11)	20/80	5	>5	>10		1.1
7 (SEQ ID NO:11)	0/100	5	2.5	5		1.8
11 (SEQ ID NO:15)	100/0	>10	2.5	2.5		0.0
11 (SEQ ID NO:15)	70/30	10	2.5	2		0.0
11 (SEQ ID NO:15)	20/80	10	5	2.5		0.0
11 (SEQ ID NO:15)	0/100	10	5	5		0.2

Example 9

Peptide I of the following structural formula:
X(SEQ ID NO:1)

was prepared as described in Example 1 and assayed for Minimum Inhibitory Concentration against E.coli and P.aeruginosa and for hemolytic activity as described in Example 2. The Minimum Inhibitory Concentration of Peptide I was 10 $\mu\text{g/ml}$ against E.coli and 5 $\mu\text{g/ml}$ against P.aeruginosa. Hemolytic activity of Peptide I at a concentration of 100 $\mu\text{g/ml}$ was 25.8 percent.

Example 10 - Antimicrobial Assays for Deletion Analogues

For the deletion analogues of the parent peptide further described in Examples 11 and 12, antimicrobial assays were carried out in 96-well tissue culture plates. Each well was incubated with a given microorganism (Escherichia coli, Staphylococcus epidermidis, Pseudomonas aeruginosa, or Staphylococcus

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aureus) suspended in LB medium. Upon addition of the parent peptide or its deletion analogues (dissolved in 1XPBS< pH7.0), each well contained a final cell density of 1.3×10^5 colony forming units (CFU)/ml of E.coli, or
5 3.5×10^5 CFU/ml P.aeruginosa, or 3.9×10^5 CFU/ml S. Aureus or 3.0×10^5 CFU/ml S. epidermidis. The final peptide concentrations ranged from 1.5 µg/ml to greater than 100 µg/ml.

10 Addition of peptide to the wells was defined as time zero. At twenty hours, the plates were placed in a Titertek Multiskan apparatus and the O.D. 620 determined. The plates as well as the initial inoculum were incubated at 37°C.

15 Five wells per plate contained medium alone, whereas five others contained medium plus cells. These controls were used to eliminate the possibility of medium contamination, while providing a measure of uninhibited growth of the microorganisms. The degree
20 of peptide activity was determined by comparing the deletion analogues with uninhibited growth of the control cells over a twenty-hour period. The effective growth inhibition of the deletion analogues is listed in the examples and tables below.

25 Example 11 - Parent Peptide and Deletion Analogues

A parent peptide having the following structural formula: X-(SEQ ID NO:3)-NH₂ as hereinabove described and deletion analogues thereof wherein one of
30 the amino acid residues was deleted were prepared as hereinabove described in Example 1 and tested for minimum inhibitory concentration with respect to E.coli, P.aeruginosa, S.aureus, and S.epidermidis at a concentration given in mg/ml as hereinabove described in Example 9. The minimum inhibitory concentrations are
35 listed below in Table VII. As used herein, the following

- 23 -

"Amino Acid Residue Deleted" refers to the number of the amino acid residue in the peptide which is deleted. All other residues remain the same as that of the normal peptide sequence.

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TABLE VII

Amino Acid Residue		Minimum Inhibitory Concentration ($\mu\text{g/ml}$)				
<u>Deleted</u>		<u>E.coli</u>	<u>P.Aeruginosa</u>	<u>S.epidermidis</u>	<u>S.aureus</u>	
None	(SEQ ID NO:3) (parent)	10	75	2.5	128	
1	(X(SEQ ID NO:46) -NH ₂)	16	16-32	4	32	
2	(X(SEQ ID NO:47) -NH ₂)	8-16	16-32	4	32-64	
3	(X(SEQ ID NO:48) -NH ₂)	4	8	4	32-64	
5	(X(SEQ ID NO:49) -NH ₂)	8	32	2	64-128	
7	(X(SEQ ID NO:50) -NH ₂)	4	4-8	2	64	
9	(X(SEQ ID NO:51) -NH ₂)	8	16	4	64	
11	(X(SEQ ID NO:52) -NH ₂)	64	8	2	256	
12	(X(SEQ ID NO:53) -NH ₂)	8	8	4	32	
14	(X(SEQ ID NO:54) -NH ₂)	8	8	2	32	
16	(X(SEQ ID NO:55) -NH ₂)	8	16	4	32	
18	(X(SEQ ID NO:56) -NH ₂)	16	8	2	32	

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Example 12

Analogues of the parent peptide were prepared wherein at least amino acid residues 1-6 were deleted, or where at least four amino acid residues were linked to amino acid residue 1 of the complete parent peptide structure. These analogues are hereinafter referred to as analogues 1 through 7.

Analogue 1 has the following structure:
X(SEQ ID NO:57).

Analogue 2 has the following structure:
X(SEQ ID NO:58).

Analogue 3 has the following structure:
X(SEQ ID NO:59).

Analogue 4 has the following structure:
X(SEQ ID NO:60).

Analogue 5 has the following structure:
X(SEQ ID NO:61).

Analogue 6 has the following structure:
X(SEQ ID NO:62).

Analogue 7 has the following structure:
X(SEQ ID NO:63).

These analogues were then assayed for MIC with respect to E.coli, P.aeruginosa, S.aureus and S.epidermidis. The results are given in Table VIII below:

TABLE VIIIMinimum Inhibitory Concentration (μ g/ml)

<u>Analogue</u>	<u>E. Coli</u>	<u>P. Aerugi- nosa</u>	<u>S. aureus</u>	<u>S. epi- dermidis</u>
1	>256	>256	>256	>256
2	128	64	>256	64
3	32-64	32-64	256	2
4	128	128	>256	32
5	>256	256	>256	64
6	>256	256	>256	128
7	>256	256	>256	128

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Example 13

The hemolytic activity of the parent peptide and the deletion analogues hereinabove described in Example 11 was examined with human red blood cells.

5 Serum was separated from red blood cells through centrifugation, and the blood cells were washed with phosphate buffered saline (PBS) at a pH of 7. The PBS was removed by centrifugation. The cells were then suspended in PBS to reach a concentration of 5 percent
10 cells in PBS. The peptides were dissolved in PBS and added to 0.5 ml of the red blood cell suspension to reach a final volume of 1 ml. Peptide concentrations are at 500 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, or 10 $\mu\text{g/ml}$. The samples of peptide and red blood cells are incubated for
15 one hour at 37°C. The samples are then centrifuged for five minutes. The supernatant was separated from the pellet and the optical density of the supernatant was measured at 414 nm. No hemolysis (blank) and 100 percent hemolysis were determined from suspensions of
20 cells in PBS and Triton 1 percent, respectively. Percent hemolysis was measured at peptide concentrations of 500 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$. The results are listed in Table IX below:

25

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TABLE IX

Amino Acid Residue Deleted	Percent Hemolysis Concentration of Peptide ($\mu\text{g/ml}$)			
	500	100	50	10
1 (SEQ ID NO:46)	16.2	10.1	7.6	1.1
2 (SEQ ID NO:47)	22.4	15.0	11.0	1.3
3 (SEQ ID NO:48)	17.9	5.7	5.1	0.9
5 (SEQ ID NO:49)	60.7	49.7	34.4	12.1
7 (SEQ ID NO:50)	43.7	12.6	4.1	0.8
9 (SEQ ID NO:51)	75.3	49.9	33.0	11.7
11 (SEQ ID NO:52)	4.4	0.6	0.1	0.0
12 (SEQ ID NO:53)	50.3	31.5	18.0	2.6
14 (SEQ ID NO:54)	28.3	7.1	3.0	0.3
16 (SEQ ID NO:55)	24.9	25.4	20.1	2.5
18 (SEQ ID NO:56)	23.0	11.4	7.5	1.6
None (SEQ ID NO:3) (parent)	N/A	21.3	16.1	3.4

Example 14

Analogues 1 through 7, as hereinabove described in Example 12 were assayed for hemolytic activity in accordance with the procedures described in Example 13. The results are given below in Table X.

TABLE X

Analogue	Percent Hemolysis Concentration of Peptide ($\mu\text{g/ml}$)			
	500	100	50	10
1 (SEQ ID NO:57)	0.6	0.0	0.0	0.0
2 (SEQ ID NO:58)	0.4	0.0	0.0	0.0
3 (SEQ ID NO:59)	6.4	3.4	1.6	0.5
4 (SEQ ID NO:60)	45.7	27.2	18.8	4.3
5 (SEQ ID NO:61)	35.1	21.6	18.0	3.0
6 (SEQ ID NO:62)	26.6	24.9	18.2	2.0
7 (SEQ ID NO:63)	17.7	19.4	17.8	2.5

Example 15 - Antibacterial Assay

The procedure for the following antibacterial assay is based upon the guidelines of the National Committee for Clinical Laboratory Standards, Document M7-T2, Volume 8, N . 8, 1988.

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Stock solutions of the following Peptides (SEQ ID NO:4) and (SEQ ID NO:64) through (SEQ ID NO:68) in accordance with the present invention are prepared at a concentration of 512 $\mu\text{g/ml}$ in sterile deionized distilled water and stored at -70°C .

(SEQ ID NO:64) is an analogue of the parent peptide wherein amino acid residues 1 through 5 have been deleted; (SEQ ID NO:65) is an analogue of the parent peptide wherein amino acid residues 1 through 4 have been deleted; (SEQ ID NO:66) is an analogue of the parent peptide wherein amino acid residues 1 through 3 have been deleted, (SEQ ID NO:67) is an analogue of the parent peptide wherein amino acid residues 1 through 7 have been deleted; and (SEQ ID No: 68) is an analogue of the parent peptide wherein amino acid residue 11 is substituted with a glycine residue. The peptides may be acetylated at the N-terminal as hereinabove described, such acetylation being indicated by the letter X.

The stock peptide solution was diluted in serial dilutions (1:2) down the wells of a microtiter plate so that the final concentrations of peptides in the wells were 0.25, 0.50, 1, 2, 4, 8, 16, 32, 64, 128, and 256 $\mu\text{g/ml}$. $1-5 \times 10^5$ CFUs/ml of either S. aureus ATCC 25923, E. coli ATCC 25922, or P. aeruginosa ATCC 27853 were added to the wells in full strength Mueller Hinton broth (BBL 11443) from a mid-log culture. The inoculum is standardized spectrophotometrically at 600nm and is verified by colony counts. The plates are incubated for 16-20 hours at 37°C , and the minimal inhibitory concentration (MIC) for each peptide is determined. Minimal inhibitory concentration is defined as the lowest concentration of peptide which produces a clear well in the microtiter plate. The results are given in Table XI below.

- 29 -

For purposes of explanation of Table XI below, S is the MIC of the peptide against S. aureus, P is the MIC of the peptide against P. aeruginosa, and E is the MIC of the peptide against E. coli.

5

TABLE XI

Peptide	MIC ($\mu\text{g/ml}$)		
	S	P	E
(SEQ ID NO:4)-NH ₂	32	64	32
X-(SEQ ID NO:4)-NH ₂	32	64	32
(SEQ ID NO:64)-NH ₂	32	16	32
X-(SEQ ID NO:65)-NH ₂	16	16, 32	32
X-(SEQ ID NO:66)-NH ₂	8	16	8
(SEQ ID NO:67)-NH ₂	128	128	128
X-(SEQ ID NO:68)-NH ₂	8	8	16

20

Example 16

The procedure of Example 15 was repeated for assaying the antimicrobial activity of amide-terminated peptides (SEQ. ID NO:64) through (SEQ ID. NO:67). The results are given in Table XII below.

25

Table XII

Peptide	MIC ($\mu\text{g/ml}$)		
	S	P	E
(SEQ. ID. NO.:64)-NH ₂	8	8	16
(SEQ. ID. NO.:65)-NH ₂	8	4	16
(SEQ. ID. NO.:66)-NH ₂	8	4	16
(SEQ. ID. NO.:67)-NH ₂	8	32	16, 32

35

Numerous modifications and variations of the present invention are possible in light of the above teachings, and, therefore, within the scope of the accompanying claims, the invention may be practiced other than as particularly described.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Houghten, Richard
Blondelle, Sylvie
- (ii) TITLE OF INVENTION: Amphiphilic Peptide Compositions
and Analogues Thereof
- (iii) NUMBER OF SEQUENCES: 68
- (iv) CORRESPONDENCE ADDRESS:
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 - (B) STREET: 180 North Stetson
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 - (F) ZIP: 60601
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) Operating System: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.24
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/554,422
 - (B) FILING DATE: 19-JUL-1990
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Gamson, Edward P.
 - (B) REGISTRATION NUMBER: 29,381
 - (C) REFERENCE/DOCKET NUMBER: 421250-80
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 3126165418
 - (B) TELEFAX: 3126165460

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 14 amino acids
 - (B) TYPE: amino acid

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- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated at
N-terminus.

(x) PUBLICATION INFORMATION:

- (A) AUTHOR: Houghten, R.
Ostresh, J.
- (B) JOURNAL: Bio Chromatography
- (D) VOLUME: 2
- (E) ISSUE: 2
- (F) PAGES: 80-83
- (G) DATE: 1987

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Leu Lys Leu Leu Lys Lys Leu Leu Lys
1 5 10

Lys Leu Lys Lys

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 14 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus.

(x) PUBLICATION INFORMATION:

- (A) AUTHOR: Houghten, R.
Ostresh, J.
- (B) JOURNAL: Bio Chromatography
- (D) VOLUME: 2
- (E) ISSUE: 2
- (F) PAGES: 80-83
- (G) DATE: 1987

(xi) SEQUENCE DESCRIPTION: SEQ ID : 2:

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Lys Leu Leu Lys Lys Leu Lys Lys Leu Leu
 1 5 10

Lys Lys Leu Leu

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:3:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
 1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:4:

Leu Leu Lys Lys Leu Lys Lys Leu Leu Lys
 1 5 10

Lys Leu Leu Lys Leu Leu
 15

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:5:

Xaa Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:6:

Leu Xaa Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide, and/or may be acetylated at N-terminus, Xaa is Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:7:

Leu Lys Xaa Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide, and/or may be acetylated at N-terminus, Xaa is Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:8:

Leu Lys Leu Xaa Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:9:

Leu Lys Leu Leu Xaa Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:10:

Leu Lys Leu Leu Lys Xaa Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

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(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide, and/or may be acetylated at N-terminus, Xaa is Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:11:

Leu Lys Leu Leu Lys Lys Xaa Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide, and/or may be acetylated at N-terminus, Xaa is Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:12:

Leu Lys Leu Leu Lys Lys Leu Xaa Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

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(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide, and/or may be acetylated at N-terminus, Xaa is Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:13:

Leu Lys Leu Leu Lys Lys Leu Leu Xaa Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:14:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Xaa
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide, and/or may be acetylated at N-terminus, Xaa is Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:15:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Xaa Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide, and/or may be acetylated at N-terminus, Xaa is Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:16:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Xaa Lys Leu Leu Lys Lys Leu
15

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(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:17:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Xaa Leu Leu Lys Lys Leu
 15

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:18:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Xaa Leu Lys Lys Leu
 15

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(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:19:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Xaa Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:20:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Xaa Lys Leu
15

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(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:21:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Xaa Leu
 15

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus, Xaa is
Met or methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:22:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Xaa
 15

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- (B) TYPE: amin acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:25:

Leu Lys Leu Lys Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:26:

Leu Lys Leu Leu Lys Lys Lys Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:27:

Leu Lys Leu Leu Lys Lys Leu Lys Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:28:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Lys Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptid

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(ix) FEATURE:

(D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:29:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Lys Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: May be a C-terminal amide,
and/or may be acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:30:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Lys Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: May be a C-terminal amide,
and/ r may be acetylated
at N-terminus.

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(xi) SEQUENCE DESCRIPTION:SEQ ID NO:31:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Lys
 15

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated at
N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:32:

Leu Lys Leu Lys Lys Lys Leu Leu Lys Lys
1 5 10

Lys Lys Lys Leu Leu Lys Lys Leu
 15

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated at
N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:33:

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Leu Lys Leu Arg Lys Lys Leu Leu Lys Lys
1 5 10

Arg Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:34:

Leu Lys Leu His Lys Lys Leu Leu Lys Lys
1 5 10

His Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:35:

Leu Lys Leu Ser Lys Lys Leu Leu Lys Lys
1 5 10

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Ser Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: C-terminal amide, acetylated at
N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:36:

Leu Lys Leu Met Lys Lys Leu Leu Lys Lys
1 5 10

Met Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: C-terminal amide, acetylated
at N-terminus, Xaa is methionine
sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:37:

Leu Lys Leu Xaa Lys Lys Leu Leu Lys Lys
1 5 10

Xaa Lys Lys Leu Leu Lys Lys Leu
15

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(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:38:

Lys Lys Lys Leu Leu Lys Lys Leu
15

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:39:

Met Lys Lys Leu Leu Lys Lys Leu
15

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 18 amin acids

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- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated at N-terminus, Xaa is methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:40:

Leu Lys Leu Xaa Lys Lys Leu Leu Lys Lys
1 5 10

Lys Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated at N-terminus, Xaa is methionine sulfoxide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:41:

Leu Lys Leu Lys Lys Lys Leu Leu Lys Lys
1 5 10

Xaa Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid

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- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide, may be acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:42:

Leu Lys Leu Arg Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: May be a C-terminal amide, may be acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:43:

Leu Lys Leu His Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: May be a C-terminal amide, may be acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:44:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Arg Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: May be a C-terminal amide, may be acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:45:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

His Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(ix) FEATURE:

(D) OTHER INFORMATION: C-terminal amide, acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:46:

Lys Leu Leu Lys Lys Leu Leu Lys Lys Leu
1 5 10

Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: C-terminal amide, acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:47

Leu Leu Leu Lys Lys Leu Leu Lys Lys Leu
1 5 10

Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: C-terminal amide, acetylated at N-terminus.

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(xi) SEQUENCE DESCRIPTION:SEQ ID NO:48:

Leu Lys Leu Lys Lys Leu Leu Lys Lys Leu
1 5 10

Lys Lys Leu Leu Lys Lys Leu
 15

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:49:

Leu Lys Leu Leu Lys Leu Leu Lys Lys Leu
1 5 10

Lys Lys Leu Leu Lys Lys Leu
 15

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:50:

Leu Lys Leu Leu Lys Lys Leu Lys Lys Leu
1 5 10

Lys Lys Leu Leu Lys Lys Leu
15

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: C-terminal amide, acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:51:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Leu
1 5 10

Lys Lys Leu Leu Lys Lys Leu
15

(1) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: C-terminal amide, acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:52:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

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Lys Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:53:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:54:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Lys Lys Leu
15

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(2) INFORMATION FOR SEQ ID NO:55:

(1) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:55:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, acetylated
at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:56:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
1 5 10

Leu Lys Lys Leu Leu Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 4 amin acids
(B) TYPE: amin acid

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- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: acetylated at N-terminus, may be a C-terminal amide

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:57:

Leu Lys Lys Leu

1

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: acetylated at N-terminus, may be a C-terminal amide

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:58:

Leu Lys Lys Leu Leu Lys Lys Leu

1

5

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: acetylated at N-terminus, may be a C-terminal amide

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(xi) SEQUENCE DESCRIPTION:SEQ ID NO:59:

Leu Leu Lys Lys Leu Lys Lys Leu Leu Lys
1 5 10

Lys Leu

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 22 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: acetylated at N-terminus, may be a C-terminal amide

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:60:

Leu Lys Lys Leu Leu Lys Leu Leu Lys Lys
1 5 10

Leu Leu Lys Lys Leu Lys Lys Leu Leu Lys
15 20

Lys Leu

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 26 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: acetylated at N-terminus, may be a C-terminal amide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:61:

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Leu Lys Lys Leu Leu Lys Lys Leu Leu Lys
1 5 10
Leu Leu Lys Lys Leu Leu Lys Lys Leu Lys
15 20
Lys Leu Leu Lys Lys Leu
25

(2) INFORMATION FOR SEQ ID NO:62:

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: acetylated at N-terminus, may be
a C-terminal amide.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:62:

Leu Leu Lys Lys Leu Lys Lys Leu Leu Lys
1 5 10
Lys Leu Leu Lys Leu Leu Lys Lys Leu Leu
15 20
Lys Lys Leu Lys Lys Leu Leu Lys Lys Leu
25 30

(2) INFORMATION FOR SEQ ID NO:63:

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: acetylated at N-terminus, may be
a C-terminal amide

(x1) SEQUENCE DESCRIPTION:SEQ ID NO:63:

Lys Leu Leu Lys Lys Leu
35

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: C-terminal amide, may be acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:64:

Lys Lys Leu

(2) INFORMATION FOR SEQ ID NO:65:

(1) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(D) OTHER INFORMATION: C-terminal amide, may be acetylated at N-terminus.

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(xi) SEQUENCE DESCRIPTION:SEQ ID NO:65:

Lys Lys Leu Leu Lys Lys Leu Lys Lys Leu
1 5 10

Leu Lys Lys Leu

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, may be acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:66:

Leu Lys Lys Leu Leu Lys Lys Leu Lys Lys
1 5 10

Leu Leu Lys Lys Leu
15

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, may be acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:67:

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Leu Lys Lys Leu Lys Lys Leu Leu Lys Lys
 1 5 10

Leu

(2) INFORMATION FOR SEQ ID NO:68:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 amino acids
 (B) TYPE: amino acids
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) FEATURE:

- (D) OTHER INFORMATION: C-terminal amide, may be
 acetylated at N-terminus.

(xi) SEQUENCE DESCRIPTION:SEQ ID NO: 68:

Leu Lys Leu Leu Lys Lys Leu Leu Lys Lys
 1 5 10

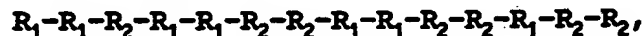
Gly Lys Lys Leu Leu Lys Lys Leu
 15

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WHAT IS CLAIMED IS;

1. A process for inhibiting growth of a target cell or virus comprising:

administering to a host a biologically active amphiphilic peptide, said peptide including the following structural formula:



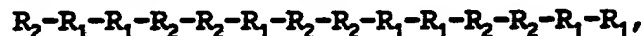
wherein $-R_1$ is a hydrophobic amino acid, and $-R_2$ is a basic hydrophilic or neutral hydrophilic amino acid, said peptide being administered in an amount effective to inhibit growth of a target cell or virus in a host.

2. The process of Claim 1 wherein said peptide has the following structural formula:

(SEQ ID NO:1).

3. A process for inhibiting growth of a target cell or virus, comprising:

administering to a host a biologically active amphiphilic peptide, said peptide including the following structural formula:



wherein R_1 is a hydrophobic amino acid and R_2 is a basic hydrophilic or neutral hydrophilic amino acid, said peptide being administered in an amount effective to inhibit growth of a target cell or virus in a host.

4. The process of Claim 3 wherein said peptide has the following structural formula:

(SEQ ID NO:2).

5. A biologically active amphiphilic peptide, said peptide being of the following structural formula:

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$R_1-R_2-R_1-R_1-R_2-R_2-R_1-R_1-R_2-R_2-R_1-R_2-R_2-R_1-R_1-R_2-R_2-R_1$,
 wherein R_1 is a hydrophobic amino acid,
 and R_2 is a basic hydrophilic or neutral hydrophilic
 amino acid.

6. The peptide of Claim 5 wherein said
 peptide has the following structural formula:
 (SEQ ID NO:3).

7. A process for inhibiting growth of a
 target cell or virus, comprising:
 administering to a host a biologically
 active amphiphilic peptide, said peptide being of the
 following structural formula:

$R_1-R_2-R_1-R_1-R_2-R_2-R_1-R_1-R_2-R_2-R_1-R_2-R_2-R_1-R_1-R_2-R_2-R_1$,
 wherein R_1 is a hydrophobic amino acid,
 and R_2 is a basic hydrophilic or neutral hydrophilic
 amino acid, said peptide being administered in an amount
 effective to inhibit growth of a target cell or virus in
 a host.

8. The process of Claim 7 wherein said
 peptide has the following structural formula:
 (SEQ ID NO:3).

9. A compound, comprising:
 an analogue of a peptide, said peptide
 being in an amide- or carboxy-terminated form, said
 peptide being represented by the following structural
 formula, and the numbers below each amino acid residue
 refer to the position of the residue in the peptide:
 LeuLysLeuLeuLysLysLeuLeuLysLysLeuLysLysLeuLeuLysLysLeu
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
 and wherein said peptide is substituted in at least n
 of positions 1, 3, 4 and 7-18 as follows:

- 66 -

<u>Residue No.</u>	<u>Substituent</u>
1	Methionine sulfoxide, Lys, or Met
3	Methionine sulfoxide, Lys, or Met
4	Methionine sulfoxide, Lys, Met, His, Ser, or Arg
7	Methionine sulfoxide, Lys, or Met
8	Methionine sulfoxide, Lys, or Met
9	Methionine sulfoxide
10	Methionine sulfoxide
11	Methionine sulfoxide, Met, Ser, Lys, Arg, His, or Gly
12	Methionine sulfoxide
13	Methionine sulfoxide, or Met
14	Methionine sulfoxide, Lys, or Met
15	Methionine sulfoxide, Lys, or Met
16	Methionine sulfoxide
17	Methionine sulfoxide
18	Methionine sulfoxide, or Met

10. The compound of Claim 9 wherein at least one of amino acid residues 1, 7, 8, 11, 14, 15, and 18 is substituted with methionine sulfoxide.

11. The compound of Claim 9 wherein at least one of amino acid residues 1, 7, 8, 14, 15, and 18 is substituted with a methionine residue.

12. The compound of Claim 9 wherein at least one of amino acid residues 4, 7, 8, 11, and 14 is substituted with a lysine residue.

13. The compound of Claim 9 wherein amino acid residue 4 is substituted with a lysine residue, and amino acid residue 11 is substituted with a methionine residue.

- 67 -

14. The compound of Claim 9 wherein at least one of amino acid residues 4 and 11 is substituted with an arginine residue.

15. The compound of Claim 9 wherein at least one of amino acid residues 4 and 11 is substituted with a histidine residue.

16. A process of inhibiting the growth of a microbe in a host, comprising:
administering to a host an effective anti-microbial amount of the compound of Claim 9.

17. A process of inhibiting the growth of a virus in a host, comprising:
administering to a host an effective anti-viral amount of the compound of Claim 9.

18. A process of inhibiting the growth of a tumor in a host, comprising:
administering to a host an effective anti-tumor amount of the compound of Claim 9.

19. A compound comprising:
an analogue of a peptide, said peptide being in an amide- or carboxy-terminated form, said peptide being represented by the following structural formula, and the numbers below each amino acid residue refer to the position of the residue in the peptide:
LeuLysLeuLeuLysLysLeuLeuLysLysLeuLysLysLeuLeuLysLysLeu
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
and wherein at least one of amino acid residues 1 through 7, 9, 11, 12, 14, 16, or 18 is deleted from said peptide.

- 68 -

20. The compound of Claim 19 wherein at least one of amino acid residues 3, 7, 11, 14 or 18 is deleted from said peptide.

21. The compound of Claim 19 wherein amino acid residues 1 through 3 are deleted from said peptide.

22. The compound of Claim 19 wherein amino acid residues 1 through 4 are deleted from said peptide.

23. The compound of Claim 19 wherein amino acid residues 1 through 5 are deleted from said peptide.

24. The compound of Claim 19 wherein amino acid residues 1 through 6 are deleted from said peptide.

25. The compound of Claim 19 wherein amino acid residues 1 through 7 are deleted from said peptide.

26. A process of inhibiting the growth of a microbe in a host, comprising: administering to a host an effective anti-microbial amount of the compound of Claim 19.

27. A process of inhibiting the growth of a virus in a host, comprising: administering to a host an effective anti-viral amount of the compound of Claim 19.

28. A process of inhibiting the growth of a tumor in a host, comprising: administering to a host an effective anti-tumor amount of the compound of Claim 19.

29. A biologically active amphiphilic peptide, said biologically active amphiphilic peptide including the following structural formula Y₁₀:

- 69 -

$R_1-R_1-R_2-R_2-R_1-R_2-R_2-R_1-R_1-R_2-R_2-R_1-R_1$,

wherein R_1 is a hydrophobic amino acid,
and R_2 is a basic hydrophilic or neutral hydrophilic
amino acid.

30. The peptide of Claim 29 wherein said
peptide includes the following structure:

$Y_{10}-Z_{10}$, wherein Y_{10} is the peptide
structure of Claim 29, and Z_{10} is:

- (i) R_2 ;
- (ii) R_2-R_1 ; or
- (iii) $R_2-R_1-R_1$.

31. The peptide of Claim 30 wherein said
peptide includes the following structural formula:

(SEQ ID NO:4).

32. A process of inhibiting the growth of a
microbe in a host, comprising:

administering to a host an effective
anti-microbial amount of the peptide of Claim 29.

33. A process of inhibiting the growth of a
virus in a host, comprising:


administering to a host an effective
anti-viral amount of the peptide of Claim 29.

34. A process of inhibiting the growth of a
tumor in a host, comprising:

administering to a host an effective
anti-tumor amount of the peptide of Claim 29.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05047

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A61K 37/02; C07K 7/08, 10 USCL: 514/12, 13; 530/326, 327		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
US	514/12, 13 530/326, 327	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
APS Text Search		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	US, A 4,507,230 (Tam et al) 26 March 1985, See abstract.	1-34
A	US, A 4,810,777 (Zasloff) 07 March 1989, See abstract.	1-34
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
11 December 1991		<div style="border: 1px solid black; padding: 2px; display: inline-block;">02 JAN 1992</div>
International Searching Authority		Signature of Authorized Officer
IEA/US		 Lester L. Lee

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Summary

Document	Pages	Printed	Missed	Copies
WO009201462	72	72	0	1
Total (1)	72	72	0	-

L33 ANSWER 12 OF 24 SCISEARCH COPYRIGHT 2003 THOMSON ISI on STN
ACCESSION NUMBER: 1998:652975 SCISEARCH
THE GENUINE ARTICLE: ZZ632
TITLE: The cytotoxicity of **cationic-peptides**
is equivalent when tested in vitro against wild-type and
MDR **tumor** cell lines
AUTHOR: **Johnstone S A (Reprint)**; West M H P; Friedland
D; **Gelmon K**; Bally M B
CORPORATE SOURCE: MICROLOGIX BIOTECH INC, VANCOUVER, BC, CANADA; BC CANC
AGCY, VANCOUVER, BC, CANADA
COUNTRY OF AUTHOR: CANADA
SOURCE: ANNALS OF ONCOLOGY, (SEP 1998) Vol. 9, Supp. [2], pp.
138-138.
Publisher: KLUWER ACADEMIC PUBL, SPUIBOULEVARD 50, PO BOX
17, 3300 AA DORDRECHT, NETHERLANDS.
ISSN: 0923-7534.
DOCUMENT TYPE: Conference; Journal
FILE SEGMENT: LIFE; CLIN
LANGUAGE: English
REFERENCE COUNT: 0

ACCESSION NUMBER: 2000:488530 CAPLUS

DOCUMENT NUMBER: 133:202657

TITLE: In vitro characterization of the anticancer activity of membrane-active cationic **peptides**. I.**Peptide**-mediated cytotoxicity and **peptide**-enhanced cytotoxic activity of doxorubicin against wild-type and p-glycoprotein over-expressing tumor cell lines

AUTHOR(S): Johnstone, Sharon A.; Gelmon, Karen; Mayer, Lawrence D.; Hancock, Robert E.; Bally, Marcel B.

CORPORATE SOURCE: Division of Medical Oncology-Advanced Therapeutics, British Columbia Cancer Agency, Vancouver, BC, Can.

SOURCE: Anti-Cancer Drug Design (2000), 15(2), 151-160

CODEN: ACDDEA; ISSN: 0266-9536

PUBLISHER: Oxford University Press

DOCUMENT TYPE: Journal

LANGUAGE: English

AB Cationic amphipathic **peptides**, such as the defensins and cecropins, induce cell death in prokaryotic and eukaryotic cells by increasing membrane permeability. Increased permeability may lead to cell lysis or, alternatively, may produce subtle changes in the membrane's barrier function that promote cell death. The in vitro cytotoxic and lytic activity of short mammalian-derived extended-helical cationic **peptides** and insect-derived alpha-helical **peptides** was measured in this study with the objective of establishing the anticancer potential of these agents. Two specific aims were addressed: (i) to assess the activity of **peptides** against non-malignant cells (sheep erythrocytes and human umbilical vein **endothelial** cells) vs. **tumor** cells; and (ii) to characterize the cytotoxic activity using multidrug-resistant tumor cell lines in the presence and absence of the anthracycline doxorubicin. Cell lysis assays demonstrated that the lytic activity of the **peptides** tested was 2->50 times more cytotoxic to tumor cells than to non-malignant cells. Further, the cytotoxic activity of these **peptides** was equiv. when tested against sensitive and multidrug-resistant cell lines. In addn. to their inherent cytotoxic activity, these membrane-active **peptides** can also augment the in vitro cytotoxic activity of doxorubicin against multidrug-resistant tumor cells.

IT 155413-75-5 185502-94-7 290818-27-8

290818-28-9 290818-29-0

RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); USES (Uses)

(peptide-mediated cytotoxicity and peptide-enhanced cytotoxic anticancer activity of doxorubicin against wild-type and p-glycoprotein over-expressing tumor cell lines)

RN 155413-75-5 CAPLUS

CN L-Lysine, L-lysyl-L-tryptophyl-L-lysyl-L-leucyl-L-phenylalanyl-L-lysyl-L-lysyl-L-isoleucylglycyl-L-isoleucylglycyl-L-alanyl-L-valyl-L-leucyl-L-lysyl-L-valyl-L-leucyl-L-threonyl-L-threonylglycyl-L-leucyl-L-prolyl-L-alanyl-L-leucyl-L-lysyl-L-leucyl-L-threonyl- (9CI) (CA INDEX NAME)

Status: Path 1 of [Dialog Information Services via Modem]

Status: Initializing TCP/IP using (UseTelnetProto 1 ServiceID pto-dialog)
Trying 31060000009999...Open

DIALOG INFORMATION SERVICES

PLEASE LOGON:

***** HHHHHHHH SSSSSSSS?

Status: Signing onto Dialog

ENTER PASSWORD:

***** HHHHHHHH SSSSSSSS? *****

Welcome to DIALOG

Status: Connected

Dialog level 02.09.15D

Last logoff: 11oct02 13:48:25

Logon file405 24oct02 10:42:31

*** ANNOUNCEMENT ***

--The following files from Cambridge Scientific Abstracts (CSA) are no longer available: 14, 28, 32, 33, 36, 37, 41, 44, 56, 61, 76, 77, 108, 117, 232, 238, 269, 293, 335. Please enter HELP CSA plus the file number to identify alternative sources of information. Example: HELP CSA14.

--File 515 D&B Dun's Electronic Business Directory is now online completely updated and redesigned. For details, see HELP NEWS 515.

--File 990 - NewsRoom now contains May 2002 to present records. File 993 - NewsRoom archive contains 2002 records from January 2002-April 2002. To search all 2002 records, BEGIN 990,993 or B NEWS2002.

--Alerts have been enhanced to allow a single Alert profile to be stored and run against multiple files. Duplicate removal is available across files and for up to 12 months. The Alert may be run according to the file's update frequency or according to a custom calendar-based schedule. There are no additional prices for these enhanced features. See HELP ALERT for more information.

--U.S. Patents Fulltext (File 654) has been redesigned with new search and display features. See HELP NEWS 654 for information.

--Connect Time joins DialUnits as pricing options on Dialog. See HELP CONNECT for information.

--CLAIMS/US Patents (Files 340,341, 942) have been enhanced with both application and grant publication level in a single record. See HELP NEWS 340 for information.

--SourceOne patents are now delivered to your email inbox as PDF replacing TIFF delivery. See HELP SOURCE1 for more information.

--Important news for public and academic libraries. See HELP LIBRARY for more information.

--Important Notice to Freelance Authors--
See HELP FREELANCE for more information

For information about the access to file 43 please see Help News43.

NEW FILES RELEASED

***Dialog NewsRoom - Current 3-4 months (File 990)

***Dialog NewsRoom - 2002 Archive (File 993)

***Dialog NewsRoom - 2001 Archive (File 994)
***Dialog NewsRoom - 2000 Archive (File 995)
***TRADEMARKSCAN-Finland (File 679)
***TRADEMARKSCAN-Norway (File 678)
***TRADEMARKSCAN-Sweden (File 675)

UPDATING RESUMED

***Delphes European Business (File 481)

RELOADED

***D&B Dun's Electronic Business Directory (File 515)
***U.S. Patents Fulltext 1976-current (File 654)
***Population Demographics (File 581)
***Kompass Western Europe (File 590)
***D&B - Dun's Market Identifiers (File 516)

REMOVED

***Chicago Tribune (File 632)
***Fort Lauderdale Sun Sentinel (File 497)
***The Orlando Sentinel (File 705)
***Newport News Daily Press (File 747)
***U.S. Patents Fulltext 1980-1989 (File 653)
***Washington Post (File 146)
***Books in Print (File 470)
***Court Filings (File 793)
***Publishers, Distributors & Wholesalers of the U.S. (File 450)
***State Tax Today (File 791)
***Tax Notes Today (File 790)
***Worldwide Tax Daily (File 792)
***ISMEC: Mechanical Engineering Abstracts (File 14)
***Oceanic Abstracts (File 28)
***METADEX: Metals Science (File 32)
***Aluminium Industry Abstracts (File 33)
***Linguistics and Language Behavior Abstracts (File 36)
***Sociological Abstracts (File 37)
***Pollution Abstracts (File 41)
***Aquatic Sciences and Fisheries Abstracts (File 44)
***ARTbibliographies Modern (File 56)
***LISA (Library & Information Science Abstracts) (File 61)
***Life Sciences Collection (File 76)
***Conference Papers Index (File 77)
***Aerospace Database (File 108)
***Water Resources Abstracts (File 117)
***Applied Social Sciences Index and Abstracts (File 232)
***Abstracts in New Technologies and Engineering (File 238)
***Materials Business File (File 269)
***Engineered Materials Abstracts (File 293)
***Ceramic Abstracts (File 335)

New document supplier

IMED has been changed to INFOTRIE (see HELP OINFOTRI)

>>> Enter BEGIN HOMEBASE for Dialog Announcements <<<
>>> of new databases, price changes, etc. <<<

KWIC is set to 50.

HIGHLIGHT set on as '*'

PICKS is set ON as an alias for 5,55,159,143,358,340,344,348,351,352,447,72,73,154,155,34
9.

*

*

SYSTEM:HOME

Cost is in DialUnits

Menu System II: D2 version 1.7.8 term=ASCII

*** DIALOG HOMEBASE(SM) Main Menu ***

Information:

1. Announcements (new files, reloads, etc.)
2. Database, Rates, & Command Descriptions

3. Help in Choosing Databases for Your Topic
4. Customer Services (telephone assistance, training, seminars, etc.)
5. Product Descriptions

Connections:

6. DIALOG(R) Document Delivery
7. Data Star(R)

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/H = Help /L = Logoff /NOMENU = Command Mode

Enter an option number to view information or to connect to an online service. Enter a BEGIN command plus a file number to search a database (e.g., B1 for ERIC).

?b picks

```
>>>          351. is unauthorized
>>>          352 is unauthorized
>>>2 of the specified files are not available
      24oct02 10:42:38 User243038 Session D108.1
          $0.00      0.175 DialUnits FileHomeBase
$0.00 Estimated cost FileHomeBase
$0.03 TELNET
$0.03 Estimated cost this search
$0.03 Estimated total session cost      0.175 DialUnits
```

SYSTEM:OS - DIALOG OneSearch

File 5:Biosis Previews(R) 1969-2002/Oct W3
(c) 2002 BIOSIS

***File 5: Alert feature enhanced for multiple files, duplicates removal, customized scheduling. See HELP ALERT.**

File 55:Biosis Previews(R) 1993-2002/Oct W3
(c) 2002 BIOSIS

***File 55: Alert feature enhanced for multiple files, duplicates removal, customized scheduling. See HELP ALERT.**

File 159:Cancerlit 1975-2002/Sep
(c) format only 2002 Dialog Corporation

File 143:Biol. & Agric. Index 1983-2002/Sep
(c) 2002 The HW Wilson Co

File 358:Current BioTech Abs 1983-2002/Sep
(c) 2002 DECHEMA

***File 358: File is now current.**

File 340:CLAIMS(R)/US Patent 1950-02/Oct 22
(c) 2002 IFI/CLAIMS(R)

***File 340: Application & grant publications are in 1 record. See HELP NEWS340 & HELP ALERTS340 for search, display & Alert info.**

File 344:Chinese Patents Abs Aug 1985-2002/Oct
(c) 2002 European Patent Office

File 348:EUROPEAN PATENTS 1978-2002/Oct W02
(c) 2002 European Patent Office

File 447:IMS Patent Focus 2002/Sep
(c) 2002 IMS Health & Affiliates

File 72:EMBASE 1993-2002/Oct W3
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***File 72: Alert feature enhanced for multiple files, duplicates removal, customized scheduling. See HELP ALERT.**

File 73:EMBASE 1974-2002/Oct W3
(c) 2002 Elsevier Science B.V.

***File 73: Alert feature enhanced for multiple files, duplicates removal, customized scheduling. See HELP ALERT.**

File 154:MEDLINE(R) 1990-2002/Oct W3

***File 154: Alert feature enhanced for multiple files, duplicates removal, customized scheduling. See HELP ALERT.**

File 155:MEDLINE(R) 1966-2002/Oct W3

***File 155: Alert feature enhanced for multiple files, duplicates removal, customized scheduling. See HELP ALERT.**

Set Items Description

?s positive positive neutral hydrophobic
S1 0 POSITIVE POSITIVE NEUTRAL HYDROPHOBIC
?s tumor derived endothelial cells
S2 4 TUMOR DERIVED ENDOTHELIAL CELLS

?rd

>>>Duplicate detection is not supported for File 340.
>>>Duplicate detection is not supported for File 344.
>>>Duplicate detection is not supported for File 348.
>>>Duplicate detection is not supported for File 447.
>>>Duplicate detection is not supported for File 349.

>>>Records from unsupported files will be retained in the RD set.

...completed examining records

S3 2 RD (unique items)

?t s3/5/all

3/5/1 (Item 1 from file: 5)

DIALOG(R)File 5:Biosis Previews(R)

(c) 2002 BIOSIS. All rts. reserv.

13184806 BIOSIS NO.: 200100391955

Anti-proliferative effects of 1alpha,25-dihydroxyvitamin D3 and vitamin D analogs on murine endothelial cells.

AUTHOR: Bernardi Ronald J(a); Modzelewski Ruth A(a); Trump Donald L(a);
Johnson Candace S(a)

AUTHOR ADDRESS: (a)University of Pittsburgh Cancer Institute, Pittsburgh,
PA**USA

JOURNAL: Proceedings of the American Association for Cancer Research Annual
Meeting 42p209 March, 2001

MEDIUM: print

CONFERENCE/MEETING: 92nd Annual Meeting of the American Association for
Cancer Research New Orleans, LA, USA March 24-28, 2001

ISSN: 0197-016X

RECORD TYPE: Citation

LANGUAGE: English

SUMMARY LANGUAGE: English

DESCRIPTORS:

MAJOR CONCEPTS: Pharmacology; Tumor Biology

BIOSYSTEMATIC NAMES: Muridae--Rodentia, Mammalia, Vertebrata, Chordata,
Animalia

ORGANISMS: mouse (Muridae)--animal model

ORGANISMS: PARTS ETC: *tumor derived endothelial cells*--drug-induced
proliferation inhibition, in-vitro culture

BIOSYSTEMATIC CLASSIFICATION (SUPER TAXA): Animals; Chordates; Mammals;
Nonhuman Mammals; Nonhuman Vertebrates; Rodents; Vertebrates

CHEMICALS & BIOCHEMICALS: 1-alpha-25-dihydroxyvitamin D-3--EB 1089
analog, ILEX 7553 analog, Ro-25-6760 analog, antineoplastic-drug, tumor
cell antiproliferative effects, vitamin-drug

MISCELLANEOUS TERMS: Meeting Abstract

CONCEPT CODES:

00520 General Biology-Symposia, Transactions and Proceedings of
Conferences, Congresses, Review Annuals

12512 Pathology, General and Miscellaneous-Therapy (1971-)

22002 Pharmacology-General

24004 Neoplasms and Neoplastic Agents-Pathology; Clinical Aspects;
Systemic Effects

24008 Neoplasms and Neoplastic Agents-Therapeutic Agents; Therapy

32500 Tissue Culture, Apparatus, Methods and Media

BIOSYSTEMATIC CODES:

86375 Muridae

3/5/2 (Item 2 from file: 5)

DIALOG(R)File 5:Biosis Previews(R)

(c) 2002 BIOSIS. All rts. reserv.

10910351 BIOSIS NO.: 199799531496

Enhancement of cisplatin-mediated tumor cell kill by cytokine induction of nitric oxide production from tumor-derived endothelial cells.

AUTHOR: Modzelewski R A; Chang M-J; Phillips S L; Johnson C S

AUTHOR ADDRESS: Univ. Pittsburgh, PCI, Pittsburgh, PA 15213**USA

JOURNAL: Proceedings of the American Association for Cancer Research Annual Meeting 38 (0):p312 1997

CONFERENCE/MEETING: Eighty-eighth Annual Meeting of the American Association for Cancer Research San Diego, California, USA April 12-16, 1997

ISSN: 0197-016X

RECORD TYPE: Citation

LANGUAGE: English

REGISTRY NUMBERS: 15663-27-1: CISPLATIN; 10102-43-9: NITRIC OXIDE

DESCRIPTORS:

MAJOR CONCEPTS: Blood and Lymphatics (Transport and Circulation); Cell Biology; Endocrine System (Chemical Coordination and Homeostasis); Tumor Biology

BIOSYSTEMATIC NAMES: Muridae--Rodentia, Mammalia, Vertebrata, Chordata, Animalia

ORGANISMS: Muridae (Muridae)

BIOSYSTEMATIC CLASSIFICATION (SUPER TAXA): animals; chordates; mammals; nonhuman vertebrates; nonhuman mammals; rodents; vertebrates

CHEMICALS & BIOCHEMICALS: CISPLATIN; NITRIC OXIDE

MISCELLANEOUS TERMS: Meeting Abstract; ANTINEOPLASTIC-DRUG; CISPLATIN; CISPLATIN-MEDIATED TUMOR CELL KILL; CYTOKINE INDUCTION; NITRIC OXIDE ENHANCEMENT; NITRIC OXIDE PRODUCTION; PHARMACOLOGY; RIF-1 CELL LINE; TUMOR BIOLOGY; *TUMOR DERIVED ENDOTHELIAL CELLS*

CONCEPT CODES:

02506 Cytology and Cytochemistry-Animal

15008 Blood, Blood-Forming Organs and Body Fluids-Lymphatic Tissue and Reticuloendothelial System

17002 Endocrine System-General

24008 Neoplasms and Neoplastic Agents-Therapeutic Agents; Therapy

00520 General Biology-Symposia, Transactions and Proceedings of Conferences, Congresses, Review Annuals

10064 Biochemical Studies-Proteins, Peptides and Amino Acids

10069 Biochemical Studies-Minerals

BIOSYSTEMATIC CODES:

86375 Muridae

?ds

Set	Items	Description
S1	0	POSITIVE POSITIVE NEUTRAL HYDROPHOBIC
S2	4	TUMOR DERIVED ENDOTHELIAL CELLS
S3	2	RD (unique items)
?s endothelial cell peptide fragment		
S4	0	ENDOTHELIAL CELL PEPTIDE FRAGMENT
?s endothelial cell peptide		
S5	0	ENDOTHELIAL CELL PEPTIDE
?s peptide fragment		
S6	1041	PEPTIDE FRAGMENT

?rd

>>>Duplicate detection is not supported for File 340.

>>>Duplicate detection is not supported for File 344.

>>>Duplicate detection is not supported for File 348.

>>>Duplicate detection is not supported for File 447.

>>>Duplicate detection is not supported for File 349.

>>>Records from unsupported files will be retained in the RD set.

...examined 50 records (50)

...examined 50 records (100)

...examined 50 records (150)

...examined 50 records (200)

...examined 50 records (250)

...examined 50 records (300)

...examined 50 records (350)

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...examined 50 records (400)
...examined 50 records (450)
...examined 50 records (500)
...examined 50 records (550)
...examined 50 records (600)
...examined 50 records (650)
...examined 50 records (700)
...examined 50 records (750)
...examined 50 records (800)
...examined 50 records (850)
...examined 50 records (900)
...examined 50 records (950)
...examined 50 records (1000)
...completed examining records
      S7      530  RD (unique items)
?s s7 and endothelial cells
      530  S7
      8862  ENDOTHELIAL CELLS
      S8      0  S7 AND ENDOTHELIAL CELLS
?s s7 and tumor derived
      530  S7
      6  TUMOR DERIVED
      S9      0  S7 AND TUMOR DERIVED
?ds

```

Set	Items	Description
S1	0	POSITIVE POSITIVE NEUTRAL HYDROPHOBIC
S2	4	TUMOR DERIVED ENDOTHELIAL CELLS
S3	2	RD (unique items)
S4	0	ENDOTHELIAL CELL PEPTIDE FRAGMENT
S5	0	ENDOTHELIAL CELL PEPTIDE
S6	1041	PEPTIDE FRAGMENT
S7	530	RD (unique items)
S8	0	S7 AND ENDOTHELIAL CELLS
S9	0	S7 AND TUMOR DERIVED

```

?s s7 and endothelial tumor cells
      530  S7
      4  ENDOTHELIAL TUMOR CELLS
      S10      0  S7 AND ENDOTHELIAL TUMOR CELLS
?s s7 and endothelial
      530  S7
      518352  ENDOTHELIAL
      S11      6  S7 AND ENDOTHELIAL
?rd

```

```

>>>Duplicate detection is not supported for File 340.
>>>Duplicate detection is not supported for File 344.
>>>Duplicate detection is not supported for File 348.
>>>Duplicate detection is not supported for File 447.
>>>Duplicate detection is not supported for File 349.

>>>Records from unsupported files will be retained in the RD set.
...completed examining records
      S12      6  RD (unique items)
?s s12 and cells
      6  S12
      7445392  CELLS
      S13      4  S12 AND CELLS
?t s13/5/all

```

```

13/5/1      (Item 1 from file: 72)
DIALOG(R)File 72:EMBASE
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```

11363274 EMBASE No: 2001377485

Investigation of the metabolism of substance P at the blood-brain barrier using capillary electrophoresis with laser-induced fluorescence detection
 Freed A.L.; Audus K.L.; Lunte S.M.
 Dr. S.M. Lunte, Department of Pharmaceutical Chem., Center for
 Bioanalytical Research, University of Kansas, Lawrence, KS United States

AUTHOR EMAIL: slunte@ku.edu
Electrophoresis (ELECTROPHORESIS) (Germany) 2001, 22/17 (3778-3784)
CODEN: ELCTD ISSN: 0173-0835
DOCUMENT TYPE: Journal ; Article
LANGUAGE: ENGLISH SUMMARY LANGUAGE: ENGLISH
NUMBER OF REFERENCES: 61

Substance P (SP) metabolism was investigated upon exposure to a monolayer of bovine brain microvessel *endothelial* *cells* (BBMECs), a cell culture model of the blood-brain barrier. SP was incubated with the BBMECs and its metabolism was followed as a function of time over a 5-h period. The resulting samples were derivatized with naphthalene-2,3-dicarboxaldehyde (NDA)/cyanide, separated, and detected using cyclodextrin-modified electrokinetic chromatography with laser-induced fluorescence detection (CDMEKC-LIF). Upon exposure to the BBMEC monolayer, SP rapidly degraded to produce the N-terminal (1-9), (1-4) and (1-7) and C-terminal (2-11) and (3-11) fragments. These results were compared with those in an earlier report from our laboratory, where SP metabolism was investigated in vivo by microdialysis sampling in rat striatum.

DRUG DESCRIPTORS:

*substance P--endogenous compound--ec
naphthalene derivative; cyanide; cyclodextrin; *peptide fragment*
--endogenous compound--ec; substance P [1-4]--endogenous compound--ec;
substance P [1-7]--endogenous compound--ec; unclassified drug

MEDICAL DESCRIPTORS:

*protein metabolism; *capillary electrophoresis
blood brain barrier; laser; fluorescence; exposure; cattle; brain blood
vessel; microvasculature; endothelium cell; cell culture; biological model;
incubation time; time; sample; derivatization; phase separation; micellar
electrokinetic chromatography; protein degradation; amino terminal sequence
; carboxy terminal sequence; comparative study; laboratory; in vivo study;
microdialysis; corpus striatum; nonhuman; rat; controlled study; animal
tissue; article

DRUG TERMS (UNCONTROLLED): naphthalene 2,3 dicarboxaldehyde

CAS REGISTRY NO.: 33507-63-0 (substance P); 57-12-5 (cyanide); 12619-70-4 (cyclodextrin); 57468-16-3 (substance P [1-4]); 72226-88-1 (substance P [1-7])

SECTION HEADINGS:

027 Biophysics, Bioengineering and Medical Instrumentation

029 Clinical and Experimental Biochemistry

13/5/2 (Item 2 from file: 72)

DIALOG(R)File 72:EMBASE

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10660691 EMBASE No: 2000143365

Fractalkine-mediated *endothelial* cell injury by NK *cells*

Yoneda O.; Imai T.; Goda S.; Inoue H.; Yamauchi A.; Okazaki T.; Imai H.;
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Journal of Immunology (J. IMMUNOL.) (United States) 15 APR 2000, 164/8
(4055-4062)

CODEN: JOIMA ISSN: 0022-1767

DOCUMENT TYPE: Journal; Article

LANGUAGE: ENGLISH SUMMARY LANGUAGE: ENGLISH

NUMBER OF REFERENCES: 54

Endothelial *cells* (ECs) are primary targets of immunological attack, and their injury can lead to vasculopathy and organ dysfunction in vascular leak syndrome and in rejection of allografts or xenografts. A newly identified CXinf 3C-chemokine, fractalkine, expressed on activated ECs plays an important role in leukocyte adhesion and migration. In this study we examined the functional roles of fractalkine on NK cell activity and NK cell-mediated *endothelial* cell injury. Freshly separated NK *cells* expressed the fractalkine receptor (CXinf 3CR1) determined by FACS analysis

and efficiently adhered to immobilized full-length fractalkine, but not to the truncated forms of the chemokine domain or mucin domain, suggesting that fractalkine functions as an adhesion molecule on the interaction between NK *cells* and ECs. Soluble fractalkine enhanced NK cell cytolytic activity against K562 target *cells* in a dose- and time-dependent manner. This enhancement correlated well with increased granular exocytosis from NK *cells*, which was completely inhibited by the G protein inhibitor, pertussis toxin. Transfection of fractalkine cDNA into ECV304 *cells* or HUVECs resulted in increased adhesion of NK *cells* and susceptibility to NK cell-mediated cytotoxicity compared with control transfection. Moreover, both enhanced adhesion and susceptibility of fractalkine-transfected *cells* were markedly suppressed by soluble fractalkine or anti-CX3CR1 Ab. Our results suggest that fractalkine plays an important role not only in the binding of NK *cells* to *endothelial* *cells*, but also in NK cell-mediated endothelium damage, which may result in vascular injury.

DRUG DESCRIPTORS:

*fractalkine; *chemokine receptor--endogenous compound--ec
peptide fragment; pertussis toxin; complementary DNA; unclassified drug

MEDICAL DESCRIPTORS:

*endothelium injury--etiology--et; *natural killer cell mediated
cytotoxicity
immunopathogenesis; natural killer cell; endothelium cell; protein
expression; hormone receptor interaction; protein domain; concentration
response; exocytosis; DNA transfection; leukocyte adherence; human;
controlled study; human cell; article; priority journal

DRUG TERMS (UNCONTROLLED): fractalkine receptor--endogenous compound--ec
CAS REGISTRY NO.: 199619-66-4 (fractalkine); 70323-44-3 (pertussis toxin)

SECTION HEADINGS:

018 Cardiovascular Diseases and Cardiovascular Surgery
026 Immunology, Serology and Transplantation

13/5/3 (Item 3 from file: 72)

DIALOG(R)File 72:EMBASE

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07712487 EMBASE No: 1999197044

**A bacterial single-chain Fv antibody fragment that inhibits binding of
its parental anti-E-Selectin monoclonal antibody to activated human
endothelial *cells***

Casalvilla R.; Duenas M.; Ayala M.; Cruz S.; Cruz L.; Buurman W.A.;
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Journal of Biotechnology (J. BIOTECHNOL.) (Netherlands) 1999, 72/1-2
(1-12)

CODEN: JBITD ISSN: 0168-1656

PUBLISHER ITEM IDENTIFIER: S016816569900005X

DOCUMENT TYPE: Journal; Article

LANGUAGE: ENGLISH SUMMARY LANGUAGE: ENGLISH

NUMBER OF REFERENCES: 31

Using the polymerase chain reaction, we cloned, modified, and linked antibody variable (V) region coding genes from a mouse hybridoma, and produced a bacterial single-chain Fv (scFv) antibody fragment specific for E-Selectin. A vector of pBR322 origin, bearing the tryptophan promoter and the ompA bacterial signal peptide, was used to direct scFv expression to periplasm. The vector included a six-histidine coding sequence 5' to the scFv for the purification of the expressed protein using immobilized metal affinity chromatography (IMAC). We found that the V(H)-Linker-V(L) 32-33 kDa scFv remained insoluble after cellular fractionation, and transmission electron microscopy showed the new protein to be present in the periplasm as inclusion bodies. The scFv was solubilized using urea, purified using IMAC, and renatured to its active form. In a competitive enzyme-linked immunosorbent assay with activated human vein *endothelial* *cells* in the solid phase, the scFv competed for binding with the original monoclonal antibody. Copyright (C) 1999 Elsevier Science B.V.

DRUG DESCRIPTORS:

**peptide fragment*; **endothelial* leukocyte adhesion molecule 1

--endogenous compound--ec

signal peptide--endogenous compound--ec

MEDICAL DESCRIPTORS:

*antibody production

molecular cloning; polymerase chain reaction; hybridoma; in vitro study;
cell culture; gene construct; gene expression; endothelium cell; plasmid;
sequence analysis; protein purification; affinity chromatography;
transmission electron microscopy; cytochemistry; enzyme linked
immunosorbent assay; human; nonhuman; mouse; controlled study; human cell;
animal cell; article; priority journal

CAS REGISTRY NO.: 128875-25-2 (*endothelial* leukocyte adhesion molecule 1)

SECTION HEADINGS:

026 Immunology, Serology and Transplantation

037 Drug Literature Index

13/5/4 (Item 4 from file: 72)

DIALOG(R)File 72:EMBASE

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05815885 EMBASE No: 1994231553

Cyclic RGD peptide inhibits alpha4beta1 interaction with connecting segment 1 and vascular cell adhesion molecule

Cardarelli P.M.; Cobb R.R.; Nowlin D.M.; Scholz W.; Gorcsan F.; Moscinski M.; Yasuhara M.; Chiang S.-L.; Lobl T.J.

Tanabe Research Labs., 4540 Towne Centre Ct., San Diego, CA 92121 United States

Journal of Biological Chemistry (J. BIOL. CHEM.) (United States) 1994
, 269/28 (18668-18673)

CODEN: JBCHA ISSN: 0021-9258

DOCUMENT TYPE: Journal; Article

LANGUAGE: ENGLISH SUMMARY LANGUAGE: ENGLISH

The integrin supergene family includes receptors for a variety of extracellular matrix as well as cell surface proteins. Integrin alpha4 has been shown to play an important role in leukocyte adhesion and extravasation during immune and inflammatory reactions. One recognition sequence known to interact with alpha4 is the Leu-Asp-Val (LDV) site contained in the connecting segment 1 region of fibronectin. Here we present evidence that shows that a conformationally restricted RGD-containing peptide is a potent inhibitor of cell adhesion mediated by alpha4beta1, a receptor not convincingly documented to interact with RGD peptides. This peptide, 1-adamantaneacetyl-Cys-Gly-Arg- Gly-Asp-Ser-Pro-Cys (disulfide bridge between residues 1-8), blocks Jurkat cell adhesion to connecting segment 1-containing peptides as well as cell adhesion to cytokine-activated *endothelial* *cells*. Adhesion of Jurkat *cells* to either vascular cell adhesion molecule-expressing *cells* or recombinant vascular cell adhesion molecule-coated plates was likewise inhibited by this peptide. Furthermore, alpha4beta1 can bind directly to a cyclic RGD peptide immobilized to Sepharose. Integrins, alpha5beta1, alphavbeta3, alphaIIb/betaIIIa, alpha2beta1, alphavbeta1, alphavbeta5, alphavbeta6, and alpha3beta1, have been shown to recognize the Arg- Gly-Asp (RGD) sequence present in a variety of extracellular matrix proteins, and our data support the addition of alpha4beta1 to this group. Further studies using molecular modeling of such cyclic RGD peptides could help in the design of more potent peptides or nonpeptide mimetics that could effectively block alpha4-mediated activity and have potential application in a number of inflammatory diseases.

DRUG DESCRIPTORS:

*arginylglycylaspartic acid; *fibronectin; *integrin; *interleukin 1; *

peptide fragment; *vascular cell adhesion molecule 1

monoclonal antibody

MEDICAL DESCRIPTORS:

*cell adhesion

animal cell; article; controlled study; dna transfection; drosophila;

endothelium cell; human; human cell; leukemia cell line; nonhuman; priority journal; umbilical vein

CAS REGISTRY NO.: 99896-85-2 (arginylglycylaspartic acid); 86088-83-7 (fibronectin)

SECTION HEADINGS:

029 Clinical and Experimental Biochemistry

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WEST

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L7: Entry 3 of 5

File: DWPI

Nov 26, 2001

DERWENT-ACC-NO: 2002-062380

DERWENT-WEEK: 200222

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TITLE: Novel isolated nucleic acid molecule which encodes a fibrinogen E polypeptide which is useful for treating cancer, diabetic retinopathy, obesity, hepatitis, pneumonia, glomerulonephritis, asthma and thyroiditis

INVENTOR: LEWIS, C; STATON, C

PATENT-ASSIGNEE: BIOACTA LTD (BIOAN), UNIV SHEFFIELD (UYSHN)

PRIORITY-DATA: 2000GB-0027396 (November 9, 2000), 2000GB-0011464 (May 13, 2000), 2000GB-0014370 (June 14, 2000)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
AU 200154967 A	November 26, 2001		000	C12N015/12
WO 200188129 A1	November 22, 2001	E	041	C12N015/12

DESIGNATED-STATES: AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW AT BE CH CY DE DK EA ES FI FR GB GH GM GR IE IT KE LS LU MC MW MZ NL OA PT SD SE SL SZ TR TZ UG ZW

APPLICATION-DATA:

PUB-NO	APPL-DATE	APPL-NO	DESCRIPTOR
AU 200154967A	May 14, 2001	2001AU-0054967	
AU 200154967A		WO 200188129	Based on
WO 200188129A1	May 14, 2001	2001WO-GB02079	

INT-CL (IPC): A01 K 67/027; A61 K 38/17; C07 K 14/75; C12 N 15/12; C12 P 21/02

ABSTRACTED-PUB-NO: WO 200188129A

BASIC-ABSTRACT:

NOVELTY - An isolated nucleic acid molecule (I) which encodes a fibrinogen E polypeptide having anti-angiogenic activity, comprises a sequence which hybridizes to a 234, 240 or 186 base pair sequence, all fully defined in the specification, or a sequence which is degenerate as a result of the genetic code to it, is new.

DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for the following:

- (1) a polypeptide (II) encoded by (I);
- (2) a therapeutic composition (III) comprising (II);
- (3) a vector (IV) comprising (I);
- (4) a cell transformed/transfected (V) with (I) or (IV);
- (5) production (M1) of (III) involves purifying fibrinogen from an animal, incubating

the fibrinogen polypeptide with an effective amount of a protease capable of cleaving fibrinogen to provide at least fibrinogen E, purifying fibrinogen E from the resultant product, and formulating the product into a therapeutic composition;

(6) production of (II), comprising culturing (V) under expression conditions, and recovering the polypeptide;

(7) assembling (M2) fibrinogen E involves providing quantities of polypeptides which form fibrinogen E, providing conditions conducive to the assembly of fibrinogen E, and purifying assembled fibrinogen E from the unassembled polypeptides;

(8) a non-human transgenic animal (VI) which incorporates at least one fibrinogen gene into its genome;

(9) an imaging agent (VII) comprising (II); and

(10) a therapeutic composition (VIII) comprising (II) which is further conjugated, associated or crosslinked to an anti-angiogenic agent.

ACTIVITY - Cytostatic; antitumor; antidiabetic; ophthalmological; antiarteriosclerotic; dermatological; virucide; gynecological; anorectic; hepatotropic; antiinflammatory; nephrotropic; antiasthmatic; antithyroid.

In vivo efficacy of fibrinogen E fragment was tested on six-week-old Balb/C mice weighing 15 g. Animals were anesthetized with an intraperitoneal injection of diazepam (0.5 mg/ml) and hypnorm (fentanyl citrate 0.0315 mg/ml and fluanisone 1 mg/ml) in the ratio of 1:1 at a volume of 0.1 ml/200 g, with supplementation as required to maintain adequate anesthesia. Naive Balb/c mice were immunized subcutaneously into the right flank, following removal of the fur. Tumor cells were injected at a concentration of 3 multiply 10⁵ viable CT26 cells per animal suspended in 100 ul serum free medium. Animals were then allowed to recover. Tumor growth and animal weights were monitored daily. Tumor growth was measured daily and when the majority of animals in the cohort had tumor volumes of greater than 100 mm³ but less than 350 mm³ animals were divided into experimental and control groups. Animals then received an intraperitoneal injection of either active drug (fibrinogen E fragment 100 Mm) or vehicle (phosphate buffered saline, micro l). The results showed that tumors in the control group continued to grow steadily reaching a final tumor volume of 3072 plus or minus 255 mm³. In contrast tumors in the experimental animals had a reduced but steady rate of growth with a final tumor volume of 2052 plus or minus 414 mm³.

MECHANISM OF ACTION - Inhibitor of angiogenesis (claimed).

USE - (II) is useful for the manufacture of medicament for treating cancer, and for treating a human or an animal which would benefit from inhibition of angiogenesis or for inhibiting tumor development in a human or animal, by administering an effective amount of (II) to the human or animal, and optionally monitoring the effects of (II) on the inhibition of angiogenesis, or on the inhibition of tumor development (claimed). (II) is useful to target imaging agents, for e.g. tumors, to identify developing tumors or to monitor the effect of treatment to inhibit tumor growth. (III) is useful for modulating angiogenesis, preferably for inhibiting angiogenesis for treating ophthalmological conditions such as neovascular glaucoma, diabetic retinopathy, age-related macular degeneration, pterygium, retinopathy of prematurity, choroidal and other intraocular disorder, atherosclerosis, hemangioma, hemangioendothelioma, warts, Kaposi's sarcoma, scar keloids, allergic edema, dysfunctional uterine bleeding, follicular cysts, ovarian hyperstimulation, endometriosis, peritoneal sclerosis, adhesion formation, obesity, osteomyelitis, pannus growth, osteophyte formation, inflammatory and infectious processes (e.g. hepatitis, pneumonia, glomerulonephritis), asthma, nasal polyps, transplantation, liver regeneration, thyroiditis, thyroid enlargement, and lymphoproliferative disorders.

ABSTRACTED-PUB-NO: WO 200188129A
EQUIVALENT-ABSTRACTS:

CHOSEN-DRAWING: Dwg.0/7

DERWENT-CLASS: B04 D16 D21 P14

CPI-CODES: B04-C01G; B04-E03F; B04-E08; B04-F0100E; B04-F0200E; B04-F0700E; B04-N02A0E; B04-P0100E; B11-C07B; B14-A01; B14-A01A4; B14-E12; B14-F07; B14-G02A; B14-H01; B14-H01B; B14-K01A; B14-N03; B14-N10; B14-N11; B14-N12; B14-N15; B14-S04; D05-C12;

D05-H09; D05-H12A; D05-H12E; D05-H14; D05-H14B; D05-H16A; D05-H17A6;

(FILE 'HOME' ENTERED AT 15:41:49 ON 01 NOV 2002)

FILE 'DGENE' ENTERED AT 15:42:16 ON 01 NOV 2002

RUN GETSEQ

L1 RUN STATEMENT CREATED
RUN GETSEQ

L2 RUN STATEMENT CREATED

L3 32775 S L2 AND (TUMOR# OR TUMOUR# OR ENDOTHELI? OR ANTIBOD? OR
CANCER

L4 26402 S L2 AND (TUMOR# OR TUMOUR# OR ENDOTHELI? OR CANCER? OR
MALIGNA

L5 630 S L2 AND ENDOTHELI?

L6 541 S L5 AND (TUMOR# OR TUMOUR# OR CANCER? OR MALIGNAN?)

L7 331 S L6 AND (DETECT? OR DIAGNOS? OR IMAG? OR TARGET? OR DIRECT?)

L8 22 S L7 AND SQL<100

L9 86 S L7 AND SQL<200

L10 64 S L9 NOT L8

FILE 'DGENE' ENTERED AT 16:35:36 ON 01 NOV 2002

L11 330 S L7 NOT ABB84886/AN

L12 69 S L7 NOT (EIGHTY(W) SEVEN)/TI

L13 177 S L2 AND PY<2000

L14 59 S L13 AND PY<1999

L15 118 S L13 NOT L14

L16 12 S L15 AND SQL<51

RUN GETSEQ

L17 RUN STATEMENT CREATED

L18 27981 S L17 AND (TUMOR# OR TUMOUR# OR ENDOTHELI? OR CANCER? OR
MALIGN

L19 664 S L17 AND ENDOTHELI?

L20 565 S L19 AND (TUMOR# OR TUMOUR# OR CANCER? OR MALIGNAN?)

L21 2 S L20 AND PY<2001

L22 5673 S L17 AND SQL<51

L23 9 S L22 AND PY<1999

L24 32 S L22 AND PY<2001

L25 27 S L24 NOT L13